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THE UNIVERSITY OF ALBERTA

# CHEMICAL STUDIES OF THE METABOLITES OF *Verticicladiella*

BY

GE LIN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA

FALL 1988

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled CHEMICAL STUDIES OF THE METABOLITES OF *Verticicladiella* submitted by GE LIN in partial fulfilment of the requirememts of the degree of MASTER OF SCIENCE.

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(Supervisor)

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Date: June 15, 1988

## ABSTRACT

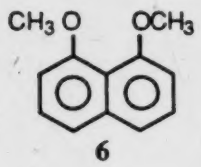
The metabolites produced when two strains of *Verticicladiella* species: NFRC C728 (Northern Forest Research Center, Canadian Forestry Service strain) and PFRC C50 (Pacific Forest Research Center, Canadian Forestry Service strain), the causative agents of the black stain root disease of many conifers, were grown on solid media (rye) as well as strain C50 was grown in liquid medium (V-8 juice) have been investigated. The metabolites produced by these two strains are quite similar.

Sixteen compounds have been isolated. Two new xanthone-type metabolites, vertixanthone (**11**) and hydroxyvertixanthone (**16**), as well as two new α-pyrone-type components, vertipyronol (**35**) and vertipyronediol (**39**) have been identified. 1-Hydroxy-8-methoxyanthraquinone (**24**) has not been reported previously from natural sources. The previously known compounds: 1,3,6,8-tetrahydroxyanthraquinone (**3**), 1,8-dimethoxynaphthalene (**6**), β-sitosterol (**8**), β-sitosteryl palmitate (**9**), palmitic acid (**10**), 5-nonadecylresorcinol (**28**), 2(3H)-benzoxazolone (**29**), isoevernin aldehyde (**30**), 3,4-dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone (**31**), 1,8-dihydroxyanthraquinone (**41**), and mycoxanthone (**42**), as well as other fatty acids and triglycerides, have also been isolated.

The structures of the metabolites were determined by spectroscopic analysis of the parent compounds and their derivatives. Comparison of spectral data with that of authentic samples and with literature values has confirmed the identity of the known compounds.

Biogenetically, the naphthalene, the anthraquinone, the xanthone, and the α-pyrone components are presumed to be formed by a polyketide pathway. The xanthones may be derived biosynthetically from anthraquinones *via* oxidative ring fission scheme already established for other fungal xanthones. Biosynthetic studies which confirm this hypothesis are reported.

The crude extracts and the anthraquinone as well as the xanthone metabolites appear to inhibit water conduction in one month old pine seedlings. The sodium salts of 1-hydroxy-8-methoxyanthraquinone (24) and 1,3,6,8-tetrahydroxyanthraquinone (3) show antibacterial activity.



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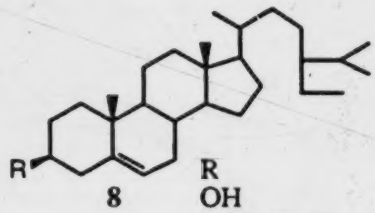
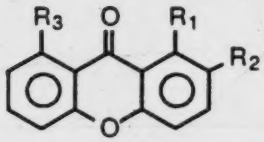
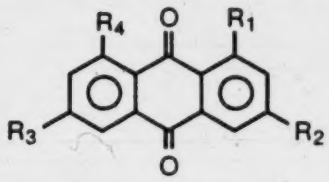


| | R |
|---|---|
| 8 | OH |
| 9 | $OCO\text{-}C_{15}H_{31}$ |

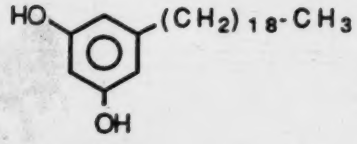
10 $CH_3\text{-}(CH_2)_{14}\text{-}COOH$



| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| 11 | $CO_2CH_3$ | H | OH |
| 15 | $CO_2CH_3$ | H | OAc |
| 16 | $CO_2CH_3$ | OH | OH |
| 20 | $CO_2CH_3$ | OAc | OAc |
| 21 | H | OH | OH |
| 42 | $CO_2CH_3$ | $OCH_3$ | OH |



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| 3 | OH | OH | OH | OH |
| 23 | OAc | OAc | OAc | OAc |
| 24 | OH | H | H | $OCH_3$ |
| 26 | $OCH_3$ | H | H | $OCH_3$ |
| 41 | OH | H | H | OH |



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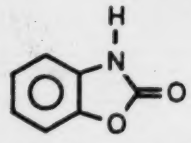

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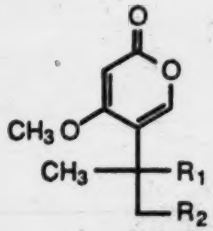
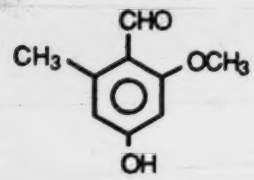


| | $R_1$ | $R_2$ |
|---|---|---|
| 35 | H | OH |
| 37 | H | OAc |
| 39 | OH | OH |
| 40 | OH | OAc |



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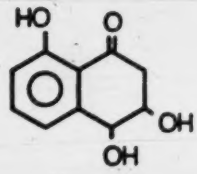

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# I. INTRODUCTION

*Verticicladiella* is the causative agent of black stain root disease of conifers. Black stain root disease, characterized by a longitudinal dark brown to black stain in the root and lower bole of conifers, is caused by the fungus, *Ceratocystis wageneri* Goheen and Cobb (anamorph *Verticicladiella wageneri* Kendrick)[1]. *Verticicladiella* is the imperfect state of the fungus. Some species of the same fungus which have formed the perfect state have been identified as *Ceratocystis* species. According to the studies by Wingfield, the genus *Verticicladiella* is synonymous with genus *Leptographium*[2].

The black stain root disease was first identified in 1938, when it was found to have killed ponderosa pine (*Pinus ponderosa* Laws) in California[3], Subsequently, it was reported on eastern white pine (*Pinus strobus* Laws) and lodgepole pine (*Pinus contorta* Dougl) in Montana[4], and on several pine species in Colorado and California[5]. Since 1971, when the disease was first reported in Washington and Oregon, it has been found with increasing frequency in Douglas fir *(Pseudotsuga menziesii* (Mirb.) Frarco) plantations in the United States[6] and in British Columbia in Canada[7].

The disease, which in Canada occurs mainly in Douglas fir, lodgepole pine and western hemlock, is debilitating to the tree and frequently results in mortality. The biology of the disease is not well known. The means by which the fungal disease kills host trees and the manner in which the disease spreads are poorly understood. Long distance spread of *Verticicladiella wageneri* probably involves root-feeding scolytid beetles or weevils[8, 9]. Once established in a tree, the fungus spreads, through root contacts or grafts, from infected to healthy trees thus producing a disease center in the stand. The fungus grows in the tracheids of infected roots, passing from cell to cell at bordered pit-pairs. On reaching the root collar, it may extend a short distance up the

bole and into uninfected roots. Extensive fungal growth in the xylem hinders water conduction, causing a vascular wilt[10]. In addition, the fungus is capable of growth through soil for a few centimeters and has been isolated from feeder roots[11].

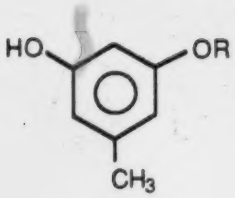
Black stain root disease damage occurs predominantly in pure, well-stocked to over-stocked lodgepole pine stands more than 50 years old (average age, 80 years old)[12]. Diagnostic of infection by *Verticicladiella* is a dark brown stain in the sapwood of root and lower stems. The symptoms of an infected tree (for example, the Douglas fir) are leader and tip growth reduction followed by foliage discoloration and crown thinning.

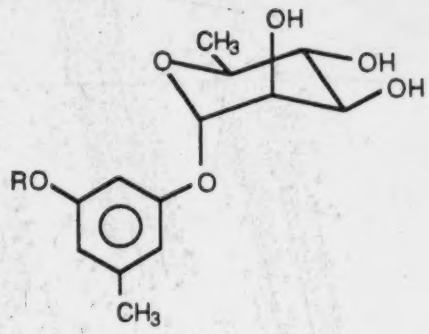
In Western North America, black stain root disease is generally attributed to *V. wageneri*, although in Western Canada two species or forms of *V. wageneri* Kendrick and *V. serpens* (Goid.) Kendrick may cause this disease[13, 14].

Our laboratories have been interested in the problem of the black stain root disease for some years. Several virulent isolates of *Verticicladiella* sp. have been studied, some of which are responsible for damage to conifers in our National Parks. We have on hand four strains of *Verticicladiella* : two from the Northern Forest Research Center, Edmonton (NFRC, Canadian Forestry Service strain C728, C713; C728=ATCC 58162. ATCC: American Type Culture Collection) and two from the Pacific Forest Research Center, Victoria (PFRC, Canadian Forestry Service strain C50, C5; C5=ATCC 42954). Isolate C50 has been identified as *Verticicladiella wageneri* (private communication, R. Hunt, PFRC), while C728 has not been fully characterized. The metabolites of strain C728, when grown in liquid medium have previously been investigated in detail[15].

In the previous study, *Verticicladiella* sp. C728 was grown in liquid still culture on 10% filtered V-8 juice containing 1% glucose. After six weeks the culture was harvested. The crude broth extract was separated by chromatography. Orcinol (1), orcinol monomethyl ether (2), 1,3,6,8-tetrahydroxyanthraquinone (3) and the α-

L-rhamnopyranosides of orcinol (4) as well as orcinol methyl ether (5) were isolated and identified. Both orcinol methyl ether and its rhamnoside show antibacterial activity, and the former also inhibits the growth of pine germlings[15].



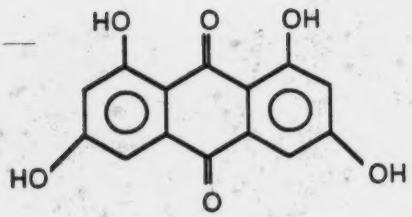




1 R = H

2 R = $CH_3$

4 R = H

5 R = $CH_3$




3

Preliminary studies in our laboratories indicated that *Verticicladiella* species produce larger quantities of metabolites when grown on solid medium and that these metabolites differed from those produced in liquid still culture. Thus the chemical investigation of the metabolites of *Verticicladiella* grown on both solid and liquid media was undertaken. Each of the four strains of *Verticicladiella* was grown on solid medium (rye) and liquid medium (V-8 juice). Both the mycelium and the broth from the liquid still culture, and the solid culture, were extracted successively with Skellysolve B, ether, methylene chloride, and ethyl acetate. Comparison of the extracts by thin layer chromatography indicated that strains C728 and C50 produce similar components in their respective extracts as do C713 and C5. However, for each single strain, there was a difference between components of solid and liquid media[16].

The objective of the work undertaken in this thesis was the isolation, separation, and structure elucidation of the metabolites produced by two strains of *Verticicladiella* : strain C728 grown on solid medium (rye) and strain C50 grown on both solid medium (rye) and liquid medium (V-8 juice). The objective was the comparison of the metabolites of C50 and C728 when grown on the same medium and when grown on different media, the identification of any biologically active components, and a study of the biogenetic origin of the metabolites.

## II. RESULTS AND DISCUSSION

### 1. Metabolites of *Verticicladiella* sp. C50 Grown on Solid Medium

#### 1.1 Isolation of the crude extracts

*Verticicladiella* species C50 was grown on solid medium consisting of moist, sterile winter rye for six weeks. The solid culture was extracted successively with Skellysolve B (SKB), ether, methylene chloride, and ethyl acetate to give four crude extracts. A blank , which was uninoculated rye, was extracted in the same way in order to compare the metabolites produced by the fungus with the compounds from the rye itself.

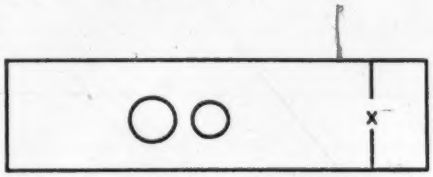
The four crude extracts were examined by thin layer chromatography (tlc). The ether extract and the ethyl acetate extract appeared to contain more components than other extracts. The metabolites of each crude extract were separated by chromatography. The pure compounds isolated from each of these four extracts are listed in Table 1. Some fatty acids and triglycerides isolated from the crude extracts were also isolated from the blank.

Table 1. Metabolites from four extracts of C50 grown on solid medium

| Extract | Compound |
|---|---|
| SKB | 6, 8 |
| $Et_2O$ | 3, 6, 9, 11, 16, 24, 28, 29 |
| $CH_2Cl_2$ | 6, 8, 9, 10, 24, 30, 31 |
| EtOAc | 3, 6, 8, 9, 11, 24, 29, 35, 39 |

## 1.2 Metabolites from the Skellysolve B extract

The tlc of the crude Skellysolve B extract revealed the presence of one major and one minor component (Figure 1).



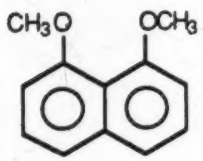

$SiO_2$ plate, Skellysolve B--ethyl acetate 3:2

Figure 1. The tlc of Skellysolve B extract

The crude extract was separated by flash chromatography over silica gel utilizing gradient elution with Skellysolve B-ethyl acetate. The major component, which was eluted with 2-4% of ethyl acetate in Skellysolve B, precipitated from the mother liquid upon concentration. Crystallization from Skellysolve B, followed by recrystallization from 95% ethanol, gave a white crystalline compound **6**.

Compound **6**, melting point 154-155°C, has a molecular formula $C_{12}H_{12}O_2$ as shown by high resolution mass spectrometry (hrms). The ultraviolet spectrum (uv) of compound **6** ($\lambda_{max}$ : 298, 310, 315, and 330 nm) shows a charaterestic naphthalene chromophore[17-19]. The $^1H$ nuclear magnetic resonance spectrum ($^1H$ nmr) displays methoxyl ($\delta$ 3.98, 6H, s) and three aromatic hydrogen signals (Figure 2) . The coupling pattern of each aromatic hydrogen signal was deduced from spin decoupling experiments (Table 3). This suggests that compound **6** is a symmetrically substituted dimethoxynaphthalene. Nuclear Overhauser enhancement (nOe) experiments indicate that the methoxyl groups are each adjacent to one hydrogen (Table 2). The spectral evidence to this point suggests that compound **6** is either 1,8-dimethoxynaphthalene (**6**) or 1,5-dimethoxynaphthalene (**7**). The $^{13}C$ nuclear

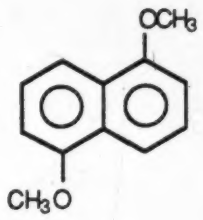
magnetic resonance spectrum ($^{13}C$ nmr) allowed us to distinguish between the two possible structures, since 1,8-dimethoxynaphthalene is expected to show seven carbon signals while six signals would be observed for 1,5-dimethoxynaphthalene. The $^{13}C$ nmr spectrum of compound **6** displays seven carbon signals and thus **6** is 1,8-dimethoxynaphthalene.




**6**

1,8-dimethoxynaphthalene

7 signals in $^{13}C$ nmr




**7**

1,5-dimethoxynaphthalene

6 signals in $^{13}C$ nmr

In order to further confirm the structure of compound **6** as 1,8-dimethoxynaphthalene, a synthetic sample was prepared by methylation of 1,8-dihydroxynaphthalene. All the spectral data for the synthetic 1,8-dimethoxy-naphthalene and compound **6** are identical. 1,8-Dimethoxynaphthalene has been isolated previously from the fungus *Daldinia cocentrica* [20]. The reported spectral data (uv, ir, $^1H$ nmr, ms) compare well with that of our metabolite[21-23].

Table 2. The nOe data for 1,8-dimethoxynaphthalene

| Signal | Irradiated | nOe (%) |
|---|---|---|
| $CH_3O$ | 3.98 | 6.86 (14.2) |
| H-2, H-7 | 6.86 | 3.98 (2.4) |

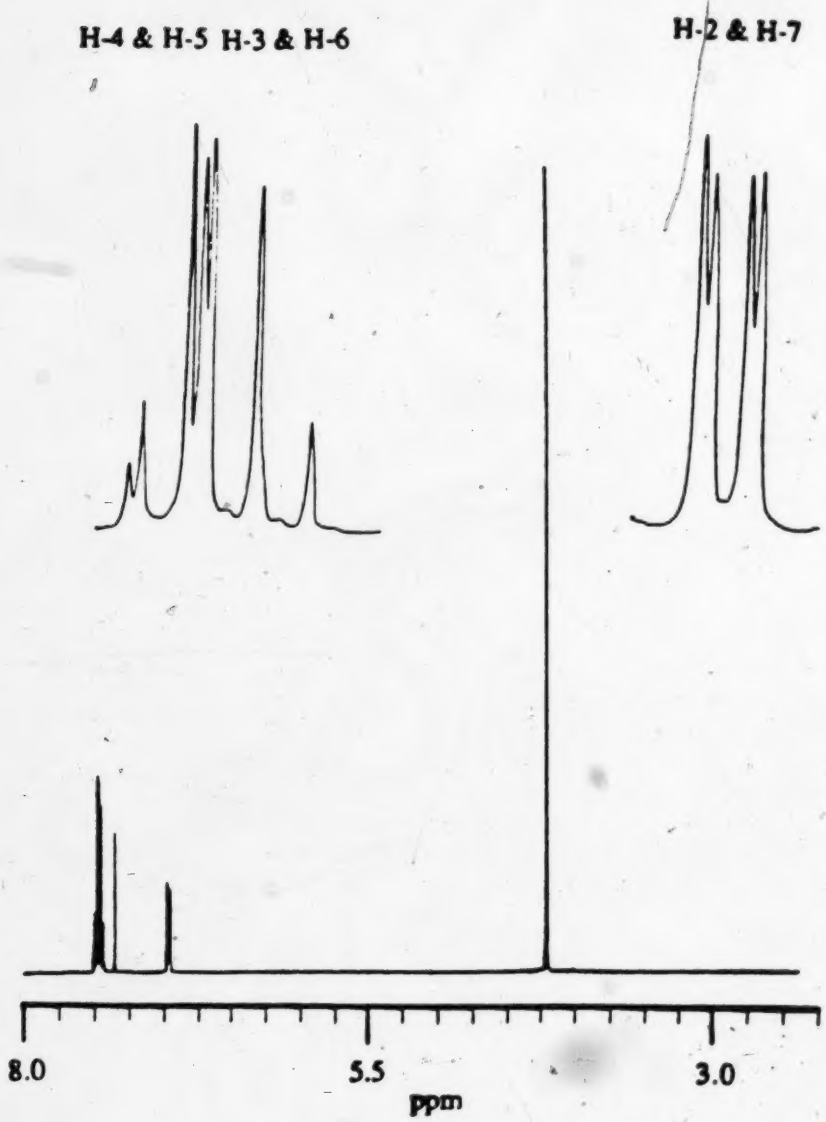
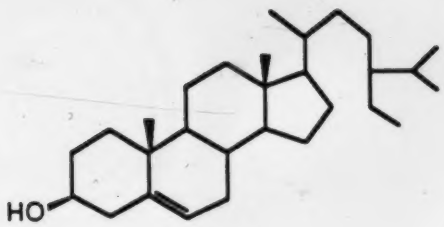



Figure 2. The $^{1}H$ nmr spectrum of 1,8-dimethoxynaphthalene ($CDCl_3$, 400 MHz)

Table 3. The spin decoupling $^1H$ nmr data for 1,8-dimethoxynaphthalene

| Signal Irradiated | Observed Change | |
|---|---|---|
| H-2, H-7 | H-3, H-6 | 7.38 t-----d (7.1 Hz) |
| 6.86 | H-4, H-5 | 7.41 dd---d (7.1 Hz) |
| H-3, H-6 | H-2, H-7 | 6.86 dd---d (1.4 Hz) |
| 7.38 | H-4, H-5 | 7.41 dd---d (1.4 Hz) |
| H-4, H-5 | H-2, H-7 | 6.86 dd---d (7.1 Hz) |
| 7.41 | H-3, H-6 | 7.38 t-----d (7.1 Hz) |

The minor component of the Skellysolve B extract, obtained by elution with 5-10% ethyl acetate in Skellysolve B, was crystallized from 95% ethanol as white needles, mp 127-128°C. Compound **8** is optically active ($[\alpha]_D$ -22.5°) and has a molecular formular $C_{29}H_{50}O$ (hrms). The fragmentation pattern in the mass spectrum of compound **8** shows loss of side chain (m/z 273, $C_{19}H_{29}O$, $M^+$ -141) and other major fragments ( m/z 399 ($M^+$ - $CH_3$), 396 ($M^+$ - $H_2O$), 381 ($M^+$ - $H_2O$ - $CH_3$), 329 ($M^+$ - $H_2O$ - $C_5H_7$), 303 ($M^+$ - $H_2O$ - $C_7H_9$), 255 ($M^+$ - side chain - $H_2O$), 231 ($M^+$ - side chain - $C_3H_6$), 213 ($M^+$ - side chain -$H_2O$ - $C_3H_6$)) suggesting that compound **8** is a steroid[24-26]. Its ir spectrum indicates the presence of a hydroxyl group (3360 $cm^{-1}$, br), while its $^1H$ nmr spectrum exhibits the hydroxyl hydrogen signal (δ 3.54, $D_2O$ exchangeable), an olefinic hydrogen (δ 5.36, m), a carbinyl hydrogen (δ 3.51, m) and six methyl hydrogen resonances. Compound **8** was identified as β-sitosterol based on its spectral characteristics and its melting point[27-31]. The spectral data of compound **8** and authentic β-sitosterol are identical.

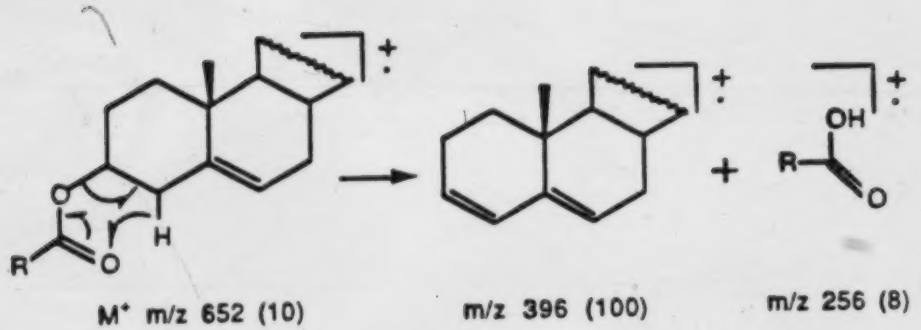



8

1.3 Metabolites from the ether extract

The crude ether extract was composed of a mixture of several compounds as indicated by tlc. Flash chromatography over silica gel using gradient elution with Skellysolve B-ethyl acetate provided a satisfactory method of separating most of the compounds from the mixture. When necessary, some fractions were further separated by fractional extraction with different solvent systems, then purified by chromatography. Chromatographic separation of the crude ether extract led to the isolation of 1,8-dimethoxynaphthalene (6) along with seven other metabolites.

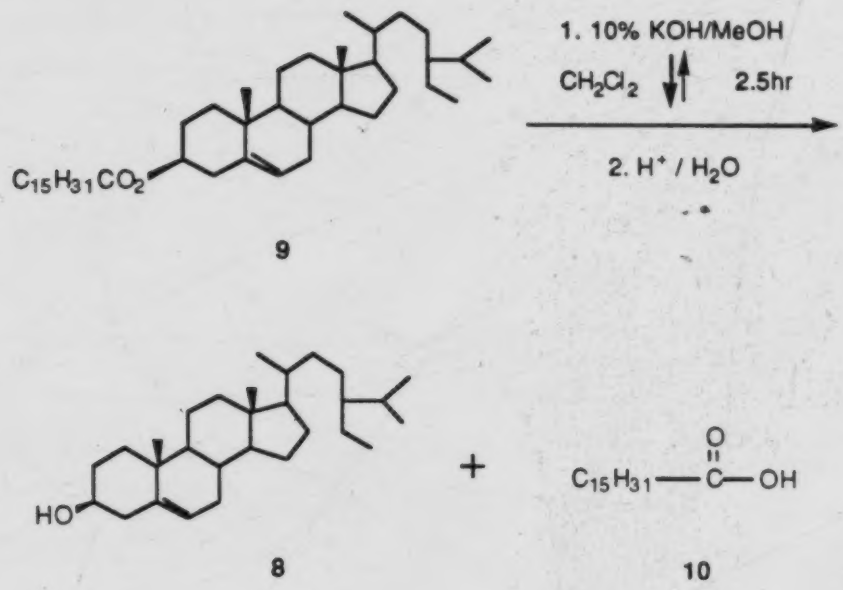
A non-polar crystalline compound **9** was isolated by elution with Skellysolve B. This compound, which is optically active ($[\alpha]_D$ -12.8°) and gives white crystalline plates, mp 92-93°C upon crystallization from acetone, has a molecular formula $C_{45}H_{80}O_2$ (hrms). Its ir spectrum reveals the presence of an ester carbonyl group (1740 $cm^{-1}$), and this is further supported by an ester carbonyl carbon resonance (δ 173.4, s) in the $^{13}C$ nmr spectrum. The mass spectrum and nuclear magnetic resonance spectra suggest that compound **9** is an ester of β-sitosterol. In its mass spectrum, the fragmentation pattern due to McLafferty rearrangment (Scheme 1) (m/z 396 ($M^+$ - 256, 100)) is consistent with the ester structure and the further loss of side chain (m/z 255 ($M^+$ - side chain - 256)) and other fragments (m/z 381 ($M^+$ - $CH_3$ - 256), 213 ($M^+$ - side chain - $C_3H_6$ - 256)) are similar to that of β-sitosterol (8)[24-26].




Scheme 1. The fragmentation of compound 9

In the $^{13}C$ nmr spectrum of compound 9, six methyl and seven methine resonances lend further support for the β-sitosterol moiety. In the $^{1}H$ nmr spectrum, the signal of the hydrogen adjacent to the ester group (δ 4.64, m) is further downfield than the carbinyl hydrogen signal (δ 3.52, m) of β-sitosterol (8) because of the ester substitution, while the chemical shift of the olefinic hydrogens of both compound 9 and β-sitosterol are the same (δ 5.36, m).

Alkaline hydrolysis of compound 9 (Scheme 2) gave two products: an alcohol and a fatty acid. The alcohol was identified as β-sitosterol (8) by comparison (tlc, ir, $^{1}H$ nmr, hrms) with an authentic sample. The fatty acid, $C_{16}H_{32}O_2$, was identified as palmitic acid (10) from its mass spectral fragmentation pattern. The high resolution mass spectrum of 10 shows the fragment corresponding to a McLafferty rearrangement (m/z 60, $C_2H_4O_2$), as well as the fragments corresponding to successive loss of 14 units characteristic of straight chain hydrocarbons. Comparison of the physical and spectral properties of the fatty acid 10 with an authentic sample of palmitic acid confirmed this identity.



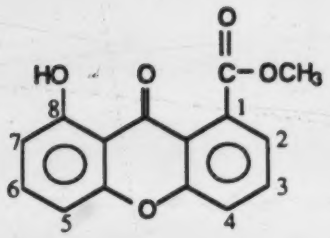

Scheme 2. Hydrolysis of compound 9

Compound **9** is thus identified as β-sitosteryl palmitate (**9**). The spectral data agree well with that reported in the literature for β-sitosteryl palmitate[32,33]. β-Sitosteryl palmitate (**9**) has been isolated previously from wheats, ryes, and several other plants[34-36]. It is interesting to note that compound **9** was obtained only from inoculated rye and was not detected in the blank extract.

A yellow compound was concentrated in the chromatographic fractions eluted with 10% ethyl acetate in Skellysolve B. Further purification by repeated flash chromatography over silica gel ( 10% ethyl acetate in Skellysolve B) and crystallization from ethyl acetate and Skellysolve B gave a pale yellow crystalline compound **11**. The structure of this compound , which is a new xanthone type metabolite and for which we propose the name vertixanthone, was determined in the following way.

The high resolution mass spectrum of vertixanthone gives the molecular formula $C_{15}H_{10}O_5$. The infrared spectrum shows hydroxyl (3100-2800 $cm^{-1}$, br) and two carbonyl (1730 and 1640 $cm^{-1}$) absorptions. The uv spectrum of vertixanthone ($\lambda_{max}$ 232, 254, 290, and 384 nm) is suggestive of a xanthone nucleus[37]. In order to further investigate the oxygen functionality of vertixanthone and to verify the presence of a xanthone skeleton, vertixanthone was acetylated (acetic anhydride, pyridine, 12 hours). The readily formed acetylvertixanthone, compound **15** ($C_{17}H_{12}O_6$), shows a new methyl resonance ($\delta$ 2.38, 3H, s) and no $D_2O$ exchangeable signal in its $^1H$ nmr spectrum. The ir spectrum of compound **15** does not show hydroxyl absorption but displays three carbonyl absorptions: an acetyl carbonyl (1764 $cm^{-1}$), an ester carbonyl (1730 $cm^{-1}$), and a normal xanthone carbonyl (1659 $cm^{-1}$)[38]. The spectral analysis of acetylvertixanthone (**15**) compared with vertixanthone suggests that vertixanthone possesses an ester group and one hydroxyl group which is hydrogen-bonded to the xanthone carbonyl.

The $^1H$ nmr spectrum of vertixanthone (Figure 3) exhibits a lowfield, $D_2O$ exchangeable signal ($\delta$ 12.24, s) attributed to a hydrogen-bonded hydroxyl hydrogen, methoxyl hydrogens ($\delta$ 4.05, 3H, s), and six aromatic hydrogen resonances as two separate AMX systems as shown by spin decoupling experiments (Table 4). Each AMX system consists of two double doublets and one apparent triplet and shows *ortho* and *meta* coupling constants (J = 8.0, 1.0 Hz). Thus, it appears that the substituents are a methoxycarbonyl group and a hydroxyl group and that the ester and the hydroxyl group are located on different 1,2,3-trisubstituted aromatic rings. There are four possible disubstituted xanthone compounds, **11-14**, which must be considered.



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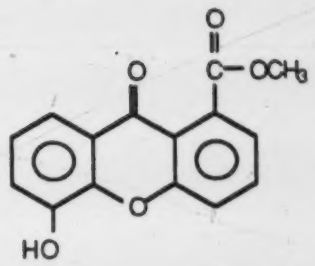

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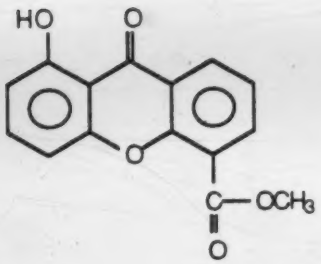

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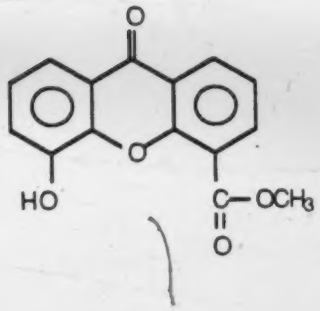

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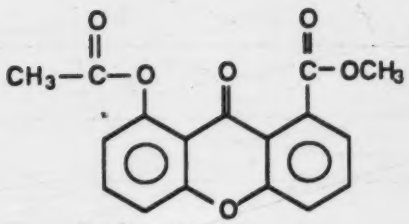

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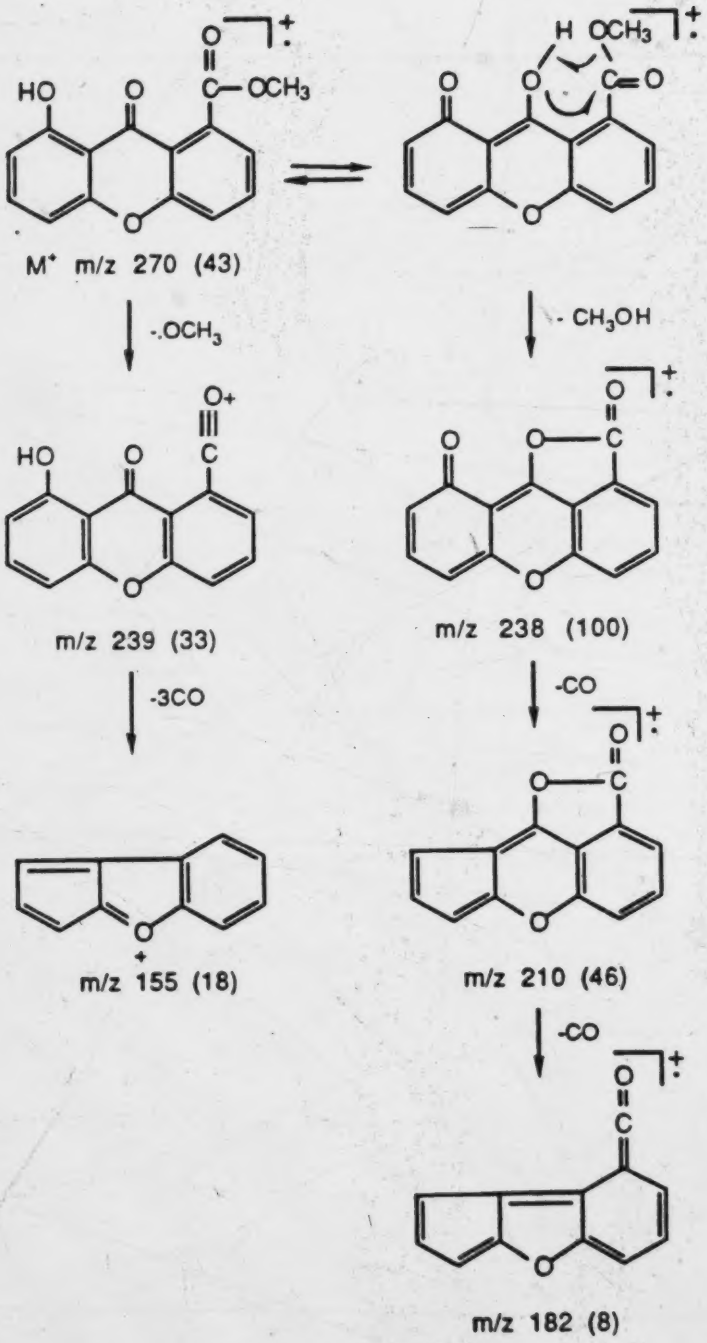
Compounds **12** and **14** are less favored since there is no intramolecular hydrogen-bonded xanthone carbonyl in the structures. The $^1H$ nmr spectrum of vertixanthone allows us to distinguish between the four possible structures. Generally, the chemical shift of a hydrogen *peri* to the carbonyl of a xanthone is observed downfield at a chemical shift greater than $\delta$ 8.00[39,40]. In the $^1H$ nmr spectrum of vertixanthone, the lowest field aromatic hydrogen resonance is observed at $\delta$ 7.65. This indicates that both the methyl ester group and the hydroxyl group are adjacent to the xanthone carbonyl, and that vertixanthone has structure **11**. Its mentioned below, structure **11** is also favored over **13** on biogenetic grounds. Therefore, the structure of vertixanthone is assigned as methyl 8-hydroxy-xanthone-1-carboxylate (**11**). The base peak in the high resolution mass spectrum of vertixanthone (**11**) corresponds to the loss of methanol (m/z 238) from the molecular ion. A tentative fragmentation scheme which accounts for the major peaks in the mass spectrum is shown in Scheme 3.

Biosynthetically, the carbon skeleton of vertixanthone may be derived from an anthraquinone *via* the degradation scheme established for other fungal xanthones[41] (see Section 6. Biosynthetic studies). These biosynthetic studies support methyl ester substitution at C-1. Biological studies show vertixanthone (**11**) inhibits water conduction in one month old pine seedlings (see Section 5. Biological studies).

Table 4. The spin decoupling $^1H$ nmr data for vertixanthone

| Signal | Irradiated | Observed | Change |
|---|---|---|---|
| H-3 | 7.77 | H-2 | 7.56 dd---d (1.0 Hz) |
| | | H-4 | 7.33 dd---d (1.0 Hz) |
| H-2 | 7.56 | H-3 | 7.77 t------d (8.0 Hz) |
| | | H-4 | 7.33 dd---d (8.0 Hz) |
| H-4 | 7.33 | H-3 | 7.77 t------d (8.0 Hz) |
| | | H-2 | 7.56 dd---d (8.0 Hz) |
| H-6 | 7.61 | H-5 | 6.94 dd---d (1.0 Hz) |
| | | H-7 | 6.28 dd---d (1.0 Hz) |
| H-5 | 6.94 | H-6 | 7.61 t------d (8.0 Hz) |
| | | H-7 | 6.28 dd---d (8.0 Hz) |
| H-7 | 6.28 | H-6 | 7.61 t------d (8.0 Hz) |
| | | H-5 | 6.94 dd---d (8.0 Hz) |




Scheme 3. The fragmentation of vertixanthone

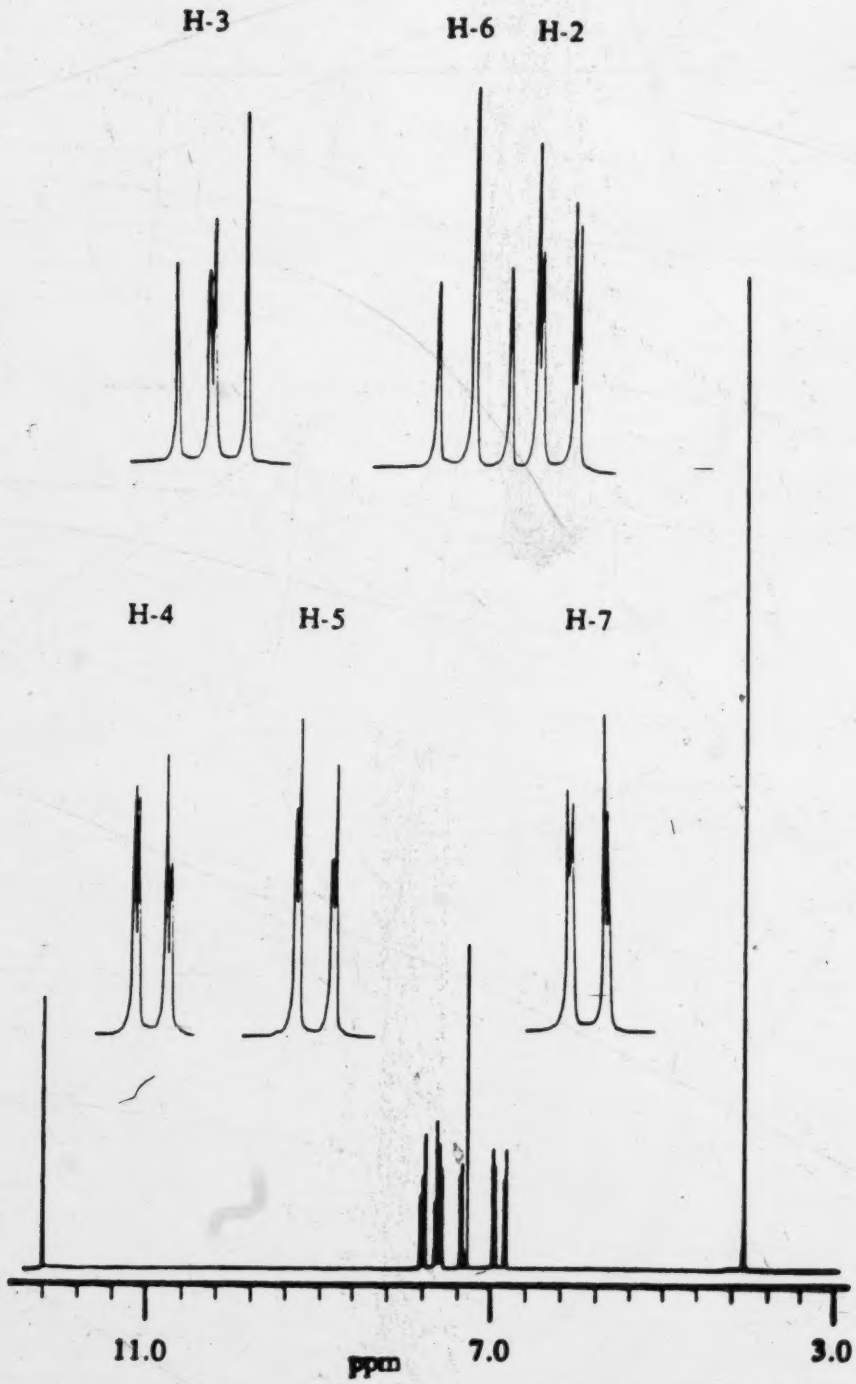


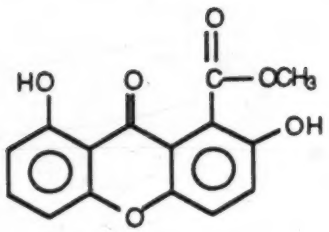

Figure 3. The $^{1}H$ nmr spectrum of vertixanthone ($CDCl_3$, 400 MHz)

The chromatographic fraction obtained with 40% ethyl acetate in Skellysolve B was composed of two components as indicated by tlc. An orange-red and a yellow pigment co-crystallized when the fraction was concentrated. Separation of these two components was achieved by fractional crystallization: the yellow component, compound **16**, was crystallized from acetone, while the orange-red pigment, compound **3**, remained in the mother liquors.

The yellow crystalline compound **16** ($C_{15}H_{10}O_6$) is another new xanthone type metabolite, for which we propose the name hydroxyvertixanthone. Its molecular formula differs from that of vertixanthone (**11**) by one oxygen atom. The ultraviolet spectrum of compound **16** (Table 6) suggests that hydroxyvertixanthone is a substituted xanthone[37] and is similar to that of other isolated xanthone type of metabolites produced by *Verticicladiella*. The infrared spectrum of compound **16** indicates the presence of hydroxyl (3300-3100 $cm^{-1}$) and two carbonyl groups (1706 and 1640 $cm^{-1}$). In order to verify the xanthone skeleton and the oxygen functionalites of compound **16**, its diacetyl derivative, compound **20**, was prepared by treatment of hydroxyvertixanthone with acetic anhydride in pyridine for 12 hours. In the ir spectrum of compound **20**, there are a typical xanthone carbonyl (1658 $cm^{-1}$) and normal ester carbonyl absorption (1734 $cm^{-1}$)[38], in addition to two acetyl ester carbonyl absorptions (1771 $cm^{-1}$, doublet). Two acetyl methyl signals (δ 2.37, 3H, s and 2.25, 3H, s) in its $^1H$ nmr spectrum (Table 7) and two acetyl carbonyl signals (δ 169.62, s and 168.77, s) in the $^{13}C$ nmr spectrum (Table 8) indicate that compound **20** is the diacetyl derivative of hydroxyvertixanthone. Therefore, hydroxyvertixanthone must have two hydroxyl groups.

The $^1H$ nmr spectrum of hydroxyvertixanthone (Figure 4) displays two downfield hydroxyl hydrogen resonances (δ 13.82, s and 10.05, s, $D_2O$ exchangeable), methoxyl hydrogens (δ 3.84, 3H, s) and five aromatic hydrogen signals (Table 7). The $^{13}C$ nmr spectrum of hydroxyvertixanthone possesses a

xanthone carbonyl (δ 180.63, s), an ester carbonyl (δ170.16, s), and a methoxyl resonance (δ 51.58, q) (Table 8). Hence, hydroxyvertixanthone is a methyl ester substituted xanthone. Spin decoupling experiments reveal that the aromatic hydrogens comprise an AB and an AMX spin systems. The AB spin system shows *ortho* coupling constants (J = 8.5, Hz) while the AMX spin system shows *ortho* and *meta* coupling constants (J = 8.0, 0.8 Hz) suggesting the presence of one 1,2,3-trisubstituted and one 1,2,3,4-tetrasubstituted aromatic ring (Table 5). The fact that the hydrogen chemical shifts of the AB system (δ 7.65, d and 7.49, d) are downfield from the hydrogen chemical shifts of both H-m and H-x in the AMX system (δ 7.07, dd and 6.80, dd) indicates that the 1,2,3,4-tetrasubsituted aromatic ring possesses the methyl ester group. Therefore, one hydroxyl and the methyl ester must be located on the tetrasubstituted ring while the other hydroxyl is on the trisubstituted ring. Comparison of the ir spectra of hydroxyvertixanthone and its diacetyl derivative reveals that the xanthone carbonyl absorption shifts from 1640 $cm^{-1}$ to 1658 $cm^{-1}$ showing hydrogen-bonding between a hydroxyl and the xanthone carbonyl in hydroxyvertixanthone. Comparison of spectral properties of hydroxyvertixanthone with those of vertixanthone (**11**) shows similar hydrogen chemical shifts for the AMX system in the $^1H$ nmr spectra and similar absorption maxima of the xanthone carbonyl (1640 $cm^{-1}$) in the ir spectra. Thus an hydroxyl substituent is present at C-8. Four possible structures for hydroxyvertixanthone, structures **16-19**, are consistent with information available to this point.



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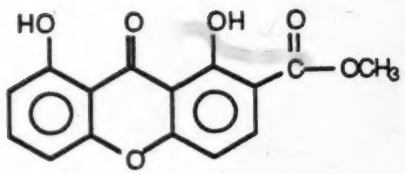

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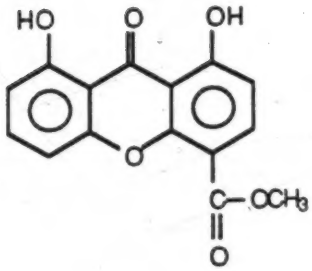
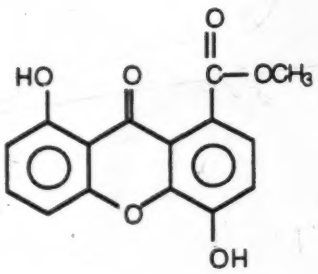


18



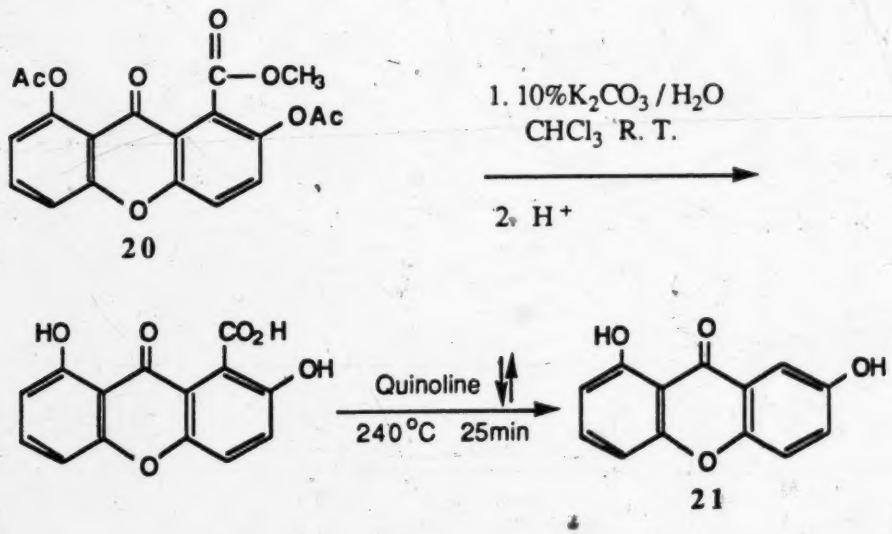
19

Table 5. The spin decoupling $^1H$ nmr data for hydroxyvertixanthone

| Signal | Irradiated | | Observed Change |
|---|---|---|---|
| H-6 | 7.73 | H-7 | 6.80 dd---d (0.8 Hz) |
| | | H-5 | 7.07 dd---d (0.8 Hz) |
| H-5 | 7.07 | H-7 | 6.80 dd---d (8.0 Hz) |
| | | H-6 | 7.73 t-----d (8.0 Hz) |
| H-7 | 6.80 | H-6 | 7.73 t-----d (8.0 Hz) |
| | | H-5 | 7.07 dd---d (8.0 Hz) |
| H-4 | 7.65 | H-3 | 7.49 d----s |
| H-3 | 7.49 | H-4 | 7.65 d----s |

In the ir spectrum of acetylhydroxyvertixanthone (**20**), the absorption of the ester carbonyl group is observed at 1734 $cm^{-1}$ compared with 1706 $cm^{-1}$ in the ir spectrum of hydroxyvertixanthone (**16**). This shift of the ester carbonyl absorption may be attributed to hydrogen-bonding and this suggests that an hydroxyl group is *ortho* to the ester group. Therefore, structures **18** and **19** may be excluded from consideration.

In order to distinguish between structures **16** and **17**, diacetylhydroxvertixanthone (**20**) was hydrolyzed ($K_2CO_3$, r.t.) then decarboxylated (quinoline, 240°C) (Scheme 4). The spectral properties (uv, ir, hrms, $^1H$ nmr ) of the reaction product compare well with that reported for the known compound, euxanthone (**21**)[42,43].




Scheme 4. Decarboxylation of hydroxyvertixanthone

The reaction product, compound **21**, has a molecular formula $C_{13}H_8O_4$. Its $^1H$ nmr spectrum is similar to that of hydroxyvertixanthone, except that in compound **21** there is no methoxyl hydrogen but another hydrogen (δ 7.62, d) which is part of a second AMX spin system. This AMX system consists of two doublets and one double of doublet with *meta* and *ortho* coupling constants (J = 3.0, 9.0 Hz) suggesting the presence of a 1,2,4-trisubstituted aromatic ring. Thus coumpound **21** is 1,7-dihydroxyxanthone. The chemical shift of H-8 in the $^1H$ nmr spectrum of compound **21** is at higher field than usual for a *peri* hydrogen due to the effect of an *ortho* hydroxyl substitution. Thus, compound **20** must be methyl 2,8-diacetoxyxanthone-1-carboxylate and it follows that hydroxyvertixanthone is methyl 2,8-dihydroxyxanthone-1-carboxylate (**16**). Compound **16** may be derived by a biosynthetic pathway similar to that proposed for vertixanthone (**11**), and this fact lends further support to the substitution of a carboxymethyl group at C-1. Hydroxyvertixanthone (**16**) inhibits water uptake in one month old pine seedlings in our biological studies (see Section 5. Biological studies).

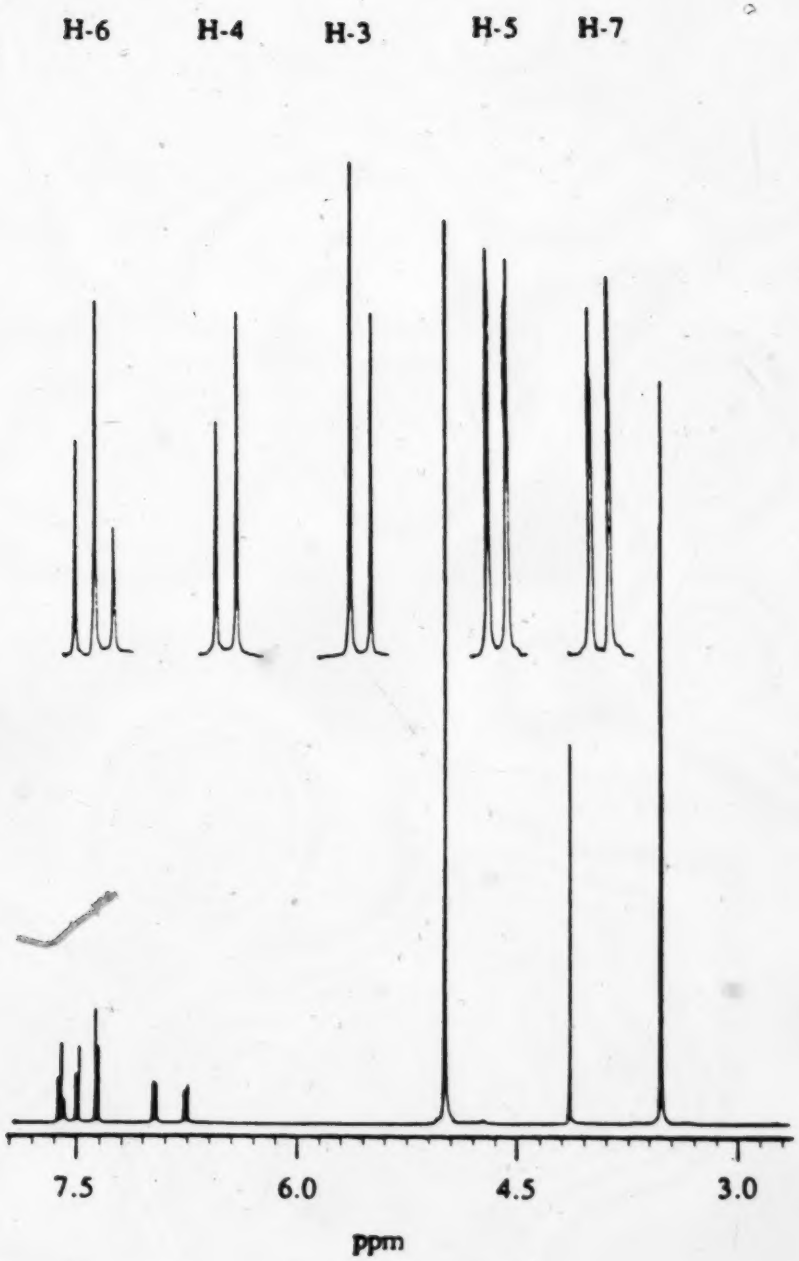
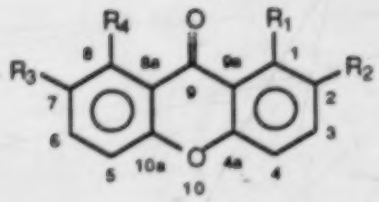



Figure 4. The $^{1}H$ nmr spectrum of hydroxyvertixanthone ($CD_3OD$, 360 MHz)



| | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| 11 | $COOCH_3$ | H | H | OH |
| 15 | $COOCH_3$ | H | H | OAc |
| 16 | $COOCH_3$ | OH | H | OH |
| 20 | $COOCH_3$ | OAc | H | OAc |
| 21 | OH | H | OH | H |
| 42 | $COOCH_3$ | $OCH_3$ | H | OH |

Table 6. The uv data for xanthone compounds

| compound | λmax nm (log ε) | | | (95% EtOH) |
|---|---|---|---|---|
| 11 | 232(4.61) | 264(4.60) | 290(4.50) | 384(3.72) |
| 16 | 238(4.14) | 264(4.25) | 290(3.71) | 390(3.50) |
| 21 | 235(4.03) | 260(4.12) | 287(3.43) | 388(3.41) |
| 42 | 235(5.13) | 261(5.16) | 290(4.60) | 385(4.52) |

Table 7. The $^1H$ nmr data for xanthone compounds

| H | Chemical Shift (mult. J in Hz) (CDCl$_3$, 360 MHz) | | | | | |
|---|---|---|---|---|---|---|
| | 11 | 15* | 16** | 20 | 21 | 42 |
| 3 | 7.77(t) (8.0) | 7.66(t) (8.5) | 7.49(d) (8.5) | 7.48(s) | 7.59(t) (8.0) | 7.42(d) (9.2) |
| 4 | 7.33(dd) (8.0,1.0) | 7.25(dd) (8.5,1.0) | 7.65(d) (8.5) | 7.48(s) | 6.93(dd) (8.0,0.8) | 7.54(d) (9.2) |
| 5 | 6.94(dd) (8.0,1.0) | 7.35(dd) (8.5,1.0) | 7.07(dd) (8.0,0.8) | 7.35(dd) (8.5,1.5) | 7.42(d) (9.0) | 6.90(dd) (8.0,1.2) |
| 6 | 7.61(t) (8.0) | 7.64(t) (8.5) | 7.73(t) (8.0) | 7.65(t) (8.5) | 7.34(dd) (9.0,3.0) | 7.58(t) (8.0) |
| R1 | 4.05(s) | 3.93(s) | 3.84(s) | 3.94 s) | 12.61(s) | 4.05(s) |
| R2 | 7.56(dd) (8.0, 1.0) | 7.48(dd) (8.5,1.0) | 10.50(br) | 2.25(s) | 6.79(dd) (8.0,0.8) | 3.92(s) |
| R3 | 6.82(dd) (8.0,1.0) | 6.95(dd) (8.5,1.0) | 6.80(dd) (8.0,0.8) | 6.95(dd) (8.5,1.5) | 8.82(br) | 6.78(dd) (8.01.2) |
| R4 | 12.24(s) | 2.38(s) | 13.82(s) | 2.37(s) | 7.62(d) (3.0) | 12.25(s) |

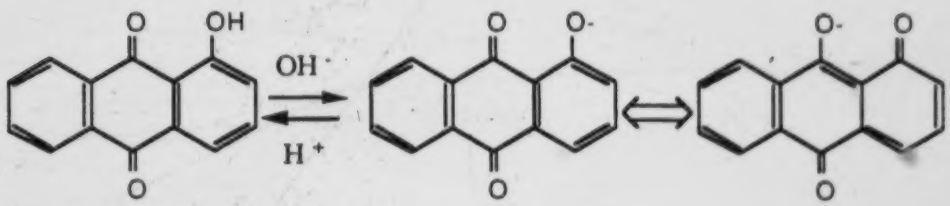
* Multiplicities by the decoupling experiments. ** In DMSO-d$_6$.

Table 8. The $^{13}C$ nmr data for xanthone compounds

| | Chemical Shift ppm (mult.) (CDCl$_3$, 75 MHz) | | | | |
|---|---|---|---|---|---|
| C | 11 | 15 | 16* | 20 | 42 |
| 1 | 133.73(s) | 134.08(s) | 116.17(s) | 125.75(s) | 120.50(s) |
| 2 | 122.72(d) | 123.00(d) | 148.00(s) | 144.50(s) | 150.21(s) |
| 3 | 135.08(d) | 134.28(d) | 125.94(d) | 129.74(d) | 120.07(d) |
| 4 | 119.53(d) | 119.38(d) | 119.04(d) | 118.75(d) | 120.00(d) |
| 5 | 111.00(d) | 118.57(d) | 109.04(d) | 119.87(d) | 110.56(d) |
| 6 | 137.20(d) | 134.76(d) | 135.83(d) | 134.95(d) | 137.12(d) |
| 7 | 106.94(d) | 116.01(d) | 106.83(d) | 116.02(d) | 106.84(d) |
| 8 | 155.94(s) | 150.11(s) | 152.25(s) | 150.12(s) | 152.51(s) |
| 9 | 181.07(s) | 174.51(s) | 180.65(s) | 174.02(s) | 181.24(s) |
| 4a | 156.11(s) | 155.27(s) | 152.58(s) | 152.75(s) | 156.09(s) |
| 8a | 109.04(s) | 115.23(s) | 107.78(s) | 113.50(s) | 108.66(s) |
| 9a | 117.58(s) | 119.71(s) | 117.21(s) | 115.51(s) | 118.26(s) |
| 10a | 161.86(s) | 156.88(s) | 160.76(s) | 156.90(s) | 161.81(s) |
| R1 | 169.60(s) | 169.69(s) | 170.16(s) | 166.85(s) | 167.60(s) |
| | 53.18(q) | 52.59(q) | 51.58(q) | 52.99(q) | 53.12(q) |
| R2 | | | | 168.77(s) | 57.05(q) |
| | | | | 20.71(q) | |
| R4 | | 169.91(s) | | 169.62(s) | |
| | | 21.21 (q) | | 21.16 (q) | |

* In CDCl$_3$ / DMSO-d$_6$

The orange-red pigment **3**, which was separated from compound **16** by fractional crystallization, has a molecular formula $C_{14}H_8O_6$ (hrms). The ultraviolet-visible spectrum (Table 9) shows absorption typical of an anthraquinone[44-46]. As well, the color of an acidic solution of compound **3** changes from yellow to red when the solution is made basic (Scheme 5). This color change is commonly observed with *peri* -hydroxyanthraquinone derivatives[47-49].



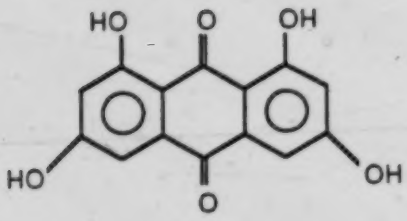

Yellow Red

Scheme 5. Chromophores of *peri* -hydroxyanthraquinone derivatives in acidic or basic solution

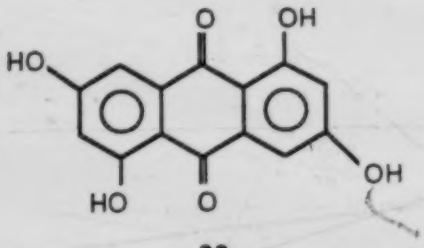
The infrared spectrum of compound **3** shows the presence of hydroxyl (3240 $cm^{-1}$), one free carbonyl (1660 $cm^{-1}$), and one hydroxgen-bonded carbonyl (1628 $cm^{-1}$). Compound **3** was acetylated with acetic anhydride in pyridine for 12 hours. The acetyl derivative, compound **23**, has molecular formula $C_{22}H_{16}O_{10}$, which differs from that of compound **3** by four acetyl units. The ir spectrum of compound **23** shows acetoxyl carbonyl absorptions (1771, 1678 $cm^{-1}$) and ketonic carbonyl absorptions (1660 $cm^{-1}$). Its $^1H$ nmr spectrum displays four aromatic hydrogen and four acetyl methyl signals (δ 2.43, 6H, s and 2.35, 6H, s) but no $D_2O$ exchangeable

signal. Compound **23** is a tetraacetyl anthraquinone and hence compound **3** must possess four hydroxyl groups.

The simplicity of the $^1H$ nmr spectrum suggests that compound **3** has a symmetrical structure. The $^1H$ nmr spectrum (Table 10) has two $D_2O$ exchangeable signals for four hydroxyl hydrogens (δ 12.24, 2H, s and 5.25, 2H, br) and two doublet signals for four aromatic hydrogens (δ 6.52, 2H, d and 7.14, 2H, d). The *meta* coupling constants for each doublet (J = 2.0 Hz) reveal the existence of two 1,2,3,5-tetrasubstituted aromatic rings. Thus, either structure **3** or structure **22** may be considered as the structure of the pigment:




**3**

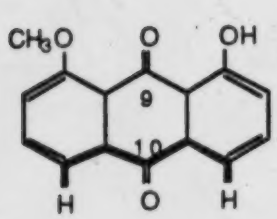


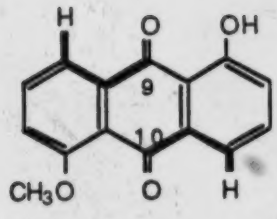

**22**

The hydroxyl substitution pattern in pigment **3** was determined from the $^{13}C$ nmr spectrum (Table 11). Two carbonyl resonances are expected for structure **3** whereas one carbonyl resonance is expected for structure **22**. The $^{13}C$ nmr spectrum of pigment **3** shows two carbonyl resonances (δ 188.50 and 182.31). Therefore, this pigment is identified as 1,3,6,8-tetrahydroxyanthraquinone (**3**). Comparison of the physical and spectral charateristics of an authentic sample of 1,3,6,8-tetrahydroxyanthraquinone with that of pigment **3** revealed its identity. 1,3,6,8-Tetrahydroxyanthraquinone has been isolated previously from *Verticicladiella* sp.

strain C728 when grown in liquid still culture[15] and also from the fungus *Aspergillus versicolor*[50].

Another anthraquinone pigment, compound **24**, was present in chromatographic fractions eluted with 20% ethyl acetate in Skellysolve B. Compound **24** was crystallized from Skellysolve B containing a few drops of ethyl acetate to give orange-yellow needles. Its structure was determined on the basis of the following observations. Compound **24** has molecular formula $C_{15}H_{10}O_4$ as shown by high resolution mass spectrometry. Its ultraviolet-visible spectrum (Table 9) suggests an anthraquinone chromophore[44-46]. The yellow color changes to red when it is dissolved in basic solution suggesting the presence of a *peri*-hydroxyl substituted anthraquinone nucleus. In the ir spectrum of compound **24**, there are one free carbonyl absorption (1668 $cm^{-1}$) and one hydrogen-bonded carbonyl absorption (1628 $cm^{-1}$) indicative of the *peri* hydroxyl substitution. Both the $^{13}C$ nmr spectrum (δ 56.69, q) and the $^1H$ nmr spectrum (δ 4.04, 3H, s) indicate the presence of a methoxyl substituent. The $^1H$ nmr spectrum of compound **24** (Figure 6) exhibits a $D_2O$ exchangeable hydroxyl hydrogen (δ 12.96, s) and six aromatic hydrogen resonances. A combination of spin decoupling experiments and difference nOe experiments allows assignment of the hydrogen coupling patterns (Table 10). There are two AMX systems with *ortho* and *meta* coupling constants (J = 7.8, 1.3 Hz) suggesting that the hydroxyl and the methoxyl group are located on different 1,2,3-trisubstituted aromatic rings. This is verified by the observation of nuclear Overhauser enchancement between the methoxyl (δ 4.04, s) and one hydrogen (δ 7.35, dd). Therefore we may consider two possible structures for this pigment: 1-hydroxy-8-methoxyanthraquinone (**24**) and 1-hydroxy-5-methoxyanthraquinone (**25**).



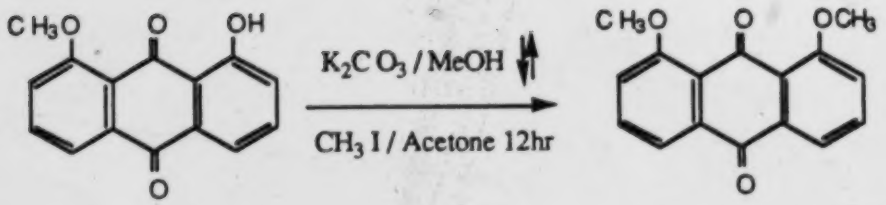




| | **24** | **25** |
|---|---|---|
| $^{13}C$ nmr | multiplicity | multiplicity |
| C-9 | singlet | doublet |
| C-10 | triplet | doublet |

Figure 5. The fully coupled $^{13}C$ nmr spectral splitting patterns expected for compounds **24** and **25**

The correct structure was derived by analysis of the fully coupled $^{13}C$ nmr spectrum. Generally, two and three bond carbon-hydrogen couplings are observed in the fully coupled $^{13}C$ nmr spectrum. Therefore, the splitting pattern of carbonyl carbons expected in structure **24** would be one triplet (C-10) and one singlet (C-9), while in structure **25** we would expect two doublets (C-9 and C-10) (Figure 5). The observed $^{13}C$ nmr spectrum of the pigment shows a triplet ($\delta$ 182.74, t, J = 4.0 Hz, C-10) and a singlet ($\delta$ 188.93, s, C-9). Thus the pigment is 1-hydroxy-8-methoxyanthraquinone (**24**).

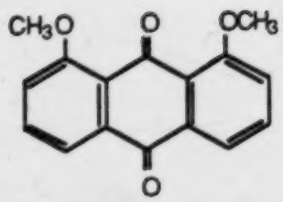
In order to further confirm the structure, 1,8-dimethoxyanthraquinone (**26**) was prepared by methylation of compound **24** ($CH_3I$, $K_2CO_3$, Scheme 6).

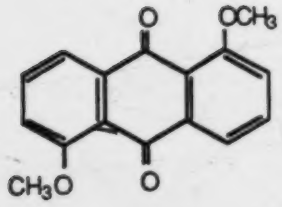



24 26

Schem 6. Methylation of 1-hydroxy-8-methoxyanthraquinone

High resolution mass spectrum of the product gives a molecular formula $C_{16}H_{12}O_4$. Its infrared spectrum shows absorptions for ketone carbonyls (1664 $cm^{-1}$ ). Its $^1H$ nmr spectrum displays one signal for two methoxyl groups (δ 3.94, 6H, s) and three signals for six aromatic hydrogens (Table 10) indicating a symmetric structure. The $^{13}C$ nmr spectrum (Table 11) shows a total of nine carbon signals including two carbonyl carbon resonances (δ 184.11, C-9 and 182.93, C-10). This further confirms a 1,8-disubstituted structure, compound **26**, since there would be eight carbon signals for a 1,5-disubstituted structure, such as compound **27**. Compound **26** is 1,8-dimethoxyanthraquinone, thus the pigment is 1-hydroxy-8-methoxyanthraquinone (**24**).




| $^{13}C$ nmr | **26** | **27** |
|---|---|---|
| Carbonyl signals | two | one |
| Total signals | nine | eight |

To the best of our knowledge, this is the first time that pigment **24** has been isolated from natural sources. Previously it was known as a synthetic compound. The physical and spectral data of compound **24** agree well with literature values for 1-hydroxy-8-methoxyanthraquinone[51-54]. 1-Hydroxy-8-methoxyanthraquinone has antibiotic activity as shown by Matsueda[55]. Its sodium salt is antibacterial to *Staphylococcus epidermidis* in our bioassay studies (see Section 5. Biological studies).

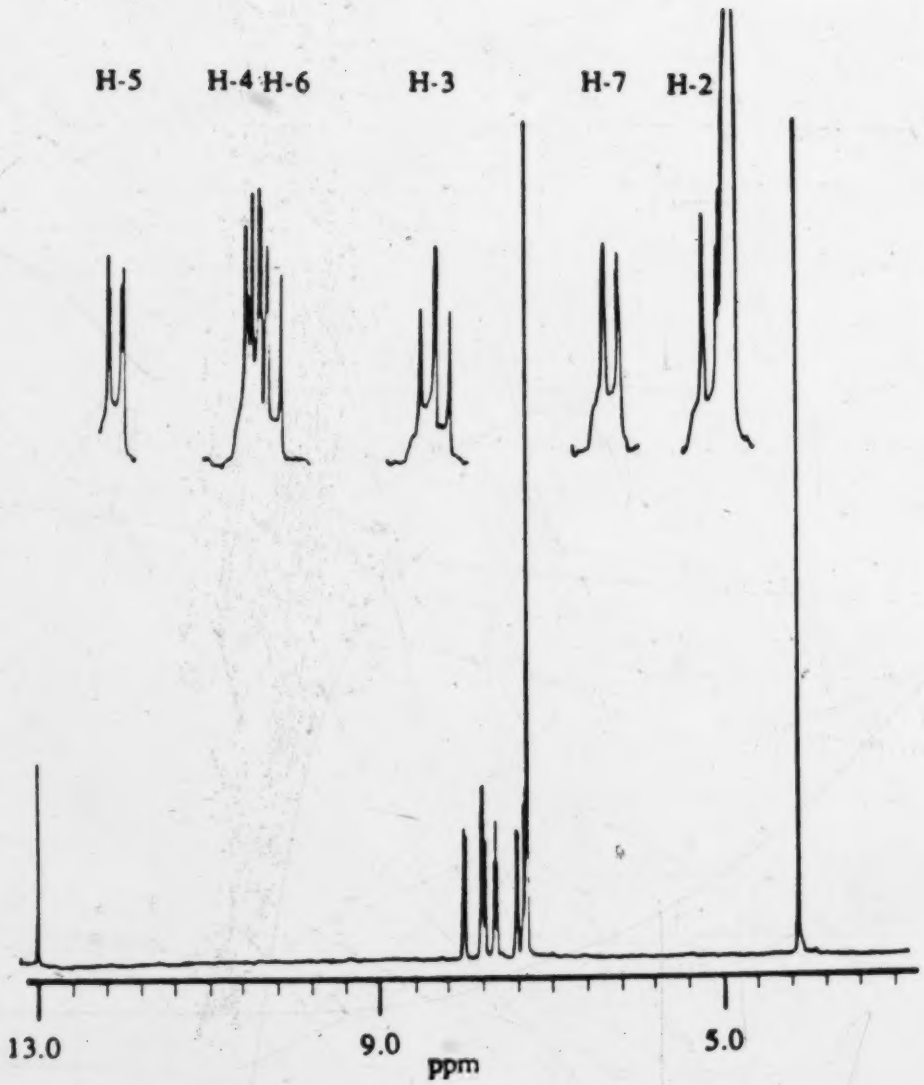
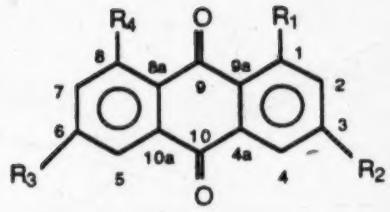



Figure 6. The $^{1}H$ nmr spectrum 1-hydroxy-8-methoxyanthraquinone ($CDCl_3$, 400 MHz)




| | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| 3 | OH | OH | OH | OH |
| 23 | OAc | OAc | OAc | OAc |
| 24 | OH | H | H | $OCH_3$ |
| 26 | $OCH_3$ | H | H | $OCH_3$ |
| 41 | OH | H | H | OH |

Table 9. The ultraviolet-visible data for anthraquinone compounds

| Compound | max nm (log ε ) | | (95% EtOH) |
|---|---|---|---|
| 3 | 252 (4.13) | 264 (4.16) | 294 (4.32) |
| | 316 (3.92) | 456 (3.18) | |
| 24 | 253 (3.92) | 277 (3.72) | 412 (3.59) |
| 41 | 252 (4.12) | 283 (3.85) | 430 (3.76) |

Table 10. The $^1H$ nmr data for anthraquinone compounds

| H | Chemical Shift (mult. J in Hz) (CDCl$_3$, 360 MHz) 3* | 23 | 24 | 26 | 41 |
|---|---|---|---|---|---|
| 2 | 6.52(d) (2.0) | 7.27(d) (2.2) | 7.29(dd) (7.8,1.3) | 7.24(dd) (8.0,1.5) | 7.30(dd) (7.5,1.8) |
| 4 | 7.41(d) (2.0) | 7.96(d) (2.2) | 7.77(dd) (7.8,1.3) | 7.68(dd) (8.0,1.5) | 7.84(dd) (7.5,1.8) |
| 5 | 7.41(d) (2.0) | 7.96(d) (2.2) | 7.96(dd) (7.8,1.3) | 7.68(dd) (8.0,1.5) | 7.84(dd) (7.5,1.8) |
| 7 | 6.52(d) (2.0) | 7.27(d) (2.2) | 7.35(dd) (7.8,1.3) | 7.24(dd) (8.0,1.5) | 7.30(dd) (7.5,1.8) |
| $R_1$ | 12.24(s) | 2.43(s) | 12.96(s) | 3.94 (s) | 12.08(s) |
| $R_2$ | 5.25 (br) | 2.35(s) | 7.69(t) (7.8) | 7.57(t) (8.0) | 7.69(t) (7.5) |
| $R_3$ | 5.25 (br) | 2.35(s) | 7.74(t) (7.8) | 7.57(t) (8.0) | 7.69(t) (7.5) |
| $R_4$ | 12.24(s) | 2.43(s) | 4.04(s) | 3.94(s) | 12.08(s) |

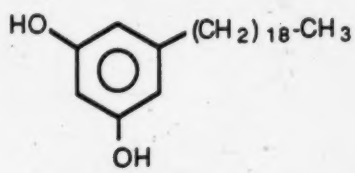
* In CDCl3-DMSO-d6.

Table 11. The $^{13}C$ nmr data for anthraquinone compounds

| Carbon | Chemical shift ppm (mult.) (CDCl$_3$, 75 MHz) 3* | 24 | 26 | 41 |
|---|---|---|---|---|
| 1 | 164.36(s) | 162.55(s) | 159.33(s) | 162.62(s) |
| 2 | 107.92(d) | 118.83(d) | 118.14(d) | 120.09(d) |
| 3 | 165.43(s) | 135.82(d) | 133.91(d) | 137.31(d) |
| 4 | 109.04(d) | 124.73(d) | 119.00(d) | 124.68(d) |
| 5 | 109.04(d) | 120.19(d) | 119.00(d) | 124.68(d) |
| 6 | 165.43(s) | 135.78(d) | 133.91(d) | 137.31(d) |
| 7 | 107.92(d) | 118.24(d) | 118.14(d) | 120.09(d) |
| 8 | 164.36(s) | 160.97(s) | 159.33(s) | 162.62(s) |
| 9 | 188.50(s) | 188.86(s) | 184.11(s) | 193.16(s) |
| 10 | 182.31(s) | 182.69(s) | 182.93(s) | 181.77(s) |
| 4a | 134.78(s) | 132.80(s) | 134.85(s) | 133.69(s) |
| 8a | 108.44(s) | 120.93(s) | 124.14(s) | 115.93(s) |
| 9a | 108.44(s) | 117.14(s) | 124.14(s) | 115.93(s) |
| 10a | 134.78(s) | 135.87(s) | 134.85(s) | 133.69(s) |
| $CH_3O$ | | 56.69(q) | 56.59(q) | |

* In MeOH-$d_4$.

Compound **28**, a colorless solid, was isolated from the chromatography fraction which was eluted with 30% ethyl acetate in Skellysolve B. The high resolution mass spectrum of compound **28** gives a molecular formula $C_{25}H_{44}O_2$ (m/z 376) and its chemical ionization mass spectrum confirms its molecular weight (m/z 377, $M^+ + 1$, 100). The base peak (m/z 124, $M^+ - C_{18}H_{38}$) and other fragments correspond to the loss of a saturated hydrocarbon unit. Its ir spectrum shows the presence of hydroxyl groups (3320-3200 $cm^{-1}$). The $^1H$ nmr spectrum displays a $D_2O$ exchangeable signal for two hydroxyl hydrogens (δ 4.73, 2H, s), aromatic hydrogen signals (δ 6.22, 2H, d and 6.15, 1H, t), and one methyl signal (δ 0.86, 3H, t). The $^{13}C$ nmr spectrum shows the methyl carbon (δ 14.16, q), six aromatic carbons, and eighteen methylene carbon signals. This reveals the presence of a straight saturated alkyl substituent, $(-(CH_2)_{18}CH_3)$. Spin decoupling experiments (Table 12) indicate that the aromatic hydrogens exhibit an $AB_2$ system with *meta* coupling constants (J = 2.0 Hz). Thus compound **28** is a 1,3,5-trisubstituted aromatic ring with two hydroxyl and a nonadecyl substituent, 5-nonadecylresorcinol. It was isolated previously from wheat bran[56]. The spectral data of compound **28** are identical with those reported. It is interesting to note that compound **28** has not been isolated from the blank rye extract.




**28**

Table 12. The spin decoupling $^1H$ nmr data for compound 28

| Signal | Irradiated | Observed | Change |
|---|---|---|---|
| H-4, H-6 | 6.22 | H-2 6.15 | t-----s |
| H-2 | 6.15 | H-4, H-6 6.22 | d---s |

The crude ether extract was composed of a mixture of components. Preliminary separation of the components into weak and strong acids was carried out in the following way. The crude extract was partitioned between 5% aqueous $NaHCO_3$ and ether. The strongly acidic components were extracted to the basic solution. The ether extract, which contains neutral and acidic components, was extracted successively with 5% $Na_2CO_3$ and 1% NaOH solutions to effect separation of the weakly acidic components into basic aqueous extracts while the less polar neutral components (B-4) remained in ether. Each of the basic extracts was neutralized with HCl and extracted with ether. In this way acidic extracts were obtained: B-1 (from 5% $NaHCO_3$), B-2 (from 5% $Na_2CO_3$), and B-3 (1% NaOH).

Purification of extract B-1 by chromatography over silica gel led to the isolation of 1,3,6,8-tetrahydroxyanthraquinone (3).

Silica gel flash chromatography of extract B-2 provided a satisfactory method for the separation of one major compound in addition to vertixanthone (11), hydroxyvertixanthone (16), and compound 3 as minor components. The major compound in extract B-2 was eluted from the chromatography column with 25% ethyl acetate in Skellysolve B. Crystallization from Skellysolve B afforded colorless crystals 29. The molecular formula ($C_7H_5NO_2$) of compound 29 was determined by

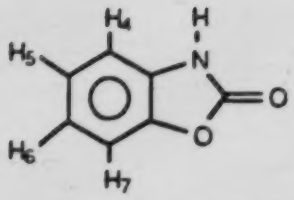
mass spectrometry (hrms: m/z 135 $M^+$; cims: m/z 153 $M^+ + 18$). The uv spectrum shows an absorption maximum at 273 nm. Addition of base (1N NaOH, 1 drop) shifts the absoption maximum to 283 nm. It returns to 273 nm upon neutralization with acid (1N HCl, 1 drop). This observation indicates the presence of an acidic hydrogen in compound **29**. The ir spectrum exhibits absorption bands characteristic of an NH group (3220 $cm^{-1}$), an amide (1620, 1478 $cm^{-1}$), and a five-membered ring lactone (1770 $cm^{-1}$)[57]. The $^1H$ nmr spectrum of compound **29** shows a $D_2O$ exchangeable NH signal ($\delta$ 8.79, s) and four aromatic hydrogen signals. The aromatic hydrogens do not display a first order spectrum. The spectrum was analyzed in the usual way and the derived coupling constants were verified using the Parameter Adjustment in NMR by Iteration Calculation (PANIC) technique[58] (Figure 7). The coupling constants of the aromatic hydrogens suggest a 1,2-disubstituted aromatic ring skeleton. The $^{13}C$ nmr spectrum of compound **29** displays a carbonyl carbon and six aromatic carbon signals (2 singlets and 4 doublets). This spectral evidence suggests that the structure of compound **29** is 2(3H)-benzoxazolone. It has been previously isolated from rye seedlings[59]. The spectral properties (ir, uv, ms, $^{13}C$ nmr) of compound **29** agree well with those reported[60,61]. 2(3H)-Benzoxazolone was not detected in the blank rye extract.

Extract B-3 contains two components as revealed by tlc. The components were separated by flash chromatography. A yellow crystalline compound, vertixanthone (**11**) and an orange-yellow pigment, 1-hydroxy-8-methoxy-anthraquinone (**24**) were isolated and identified.

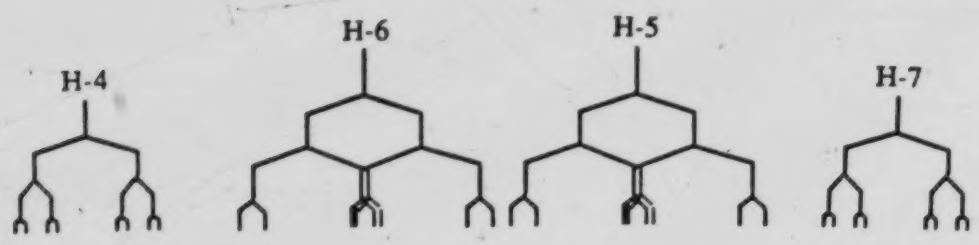
Extract B-4 contains less polar components including triglycerides and a minor compound which was identified as 5-*n*-nonadecylresorcinol (**28**).

**$^1$H-NMR** ($CDCl_3$, 400 MHz), δ

8.79 (1H, s, NH)
7.20 (1H, ddd, J = 0.7, 1.7, 7.6 Hz, H-4)
7.15 (1H, ddd, J = 1.7, 7.6, 7.6 Hz, H-6)
7.11 (1H, ddd, J = 1.7, 7.6, 7.6 Hz, H-5)
7.06 (1H, ddd, J = 0.7, 1.7, 7.6 Hz, H-7)




29



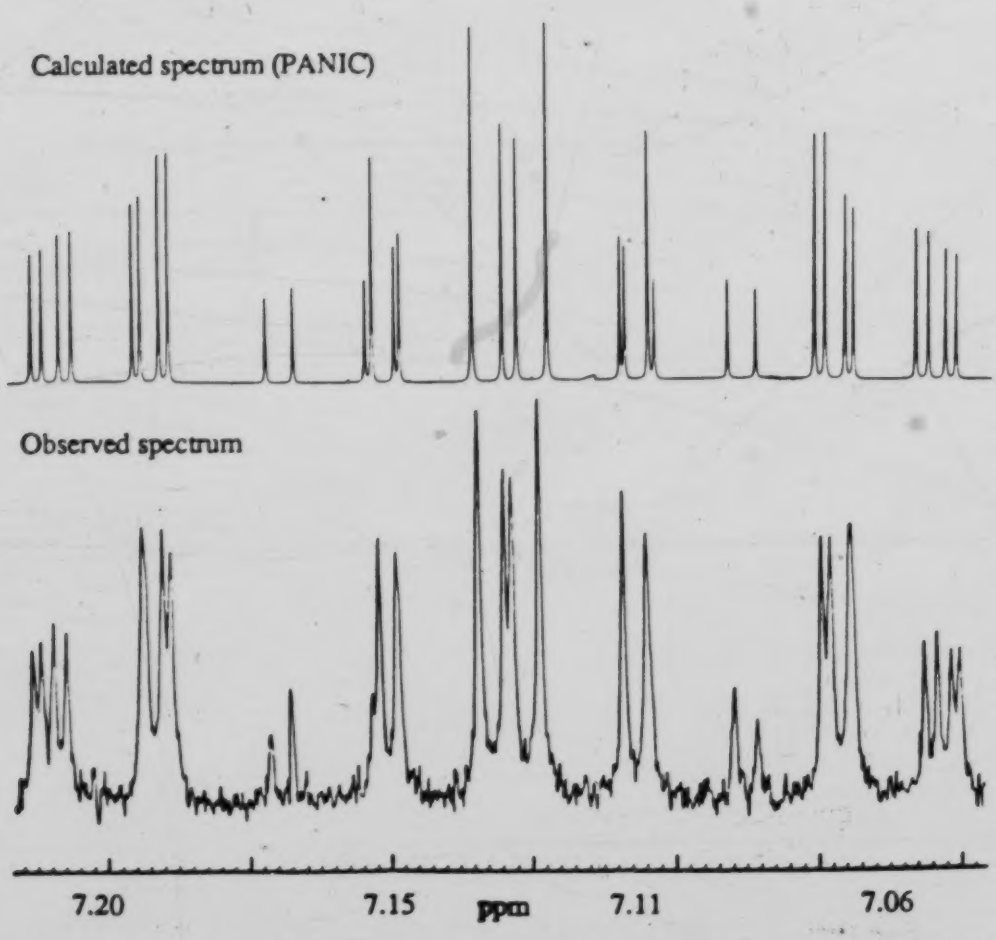



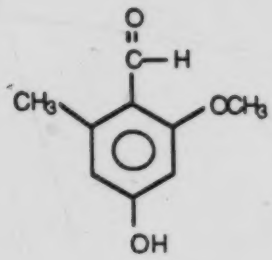

Figure 7. The $^1$H nmr spectrum of 2(3H)-benzoxazolone ($CDCl_3$, 400 MHz)

1.4 Metabolites from the methylene chloride extract.

The crude methylene chloride extract contains seven components in addition to fatty acids and triglycerides as revealed by tlc. Both the fatty acids and the triglycerides are identical with those isolated from the blank rye extract by comparison of physical and spectral properties (tlc, ms, $^1$H nmr). Silica gel flash chromatography proved to be a satisfactory method for the separation of all the components. 1,8-Dimethoxynaphthalene (**6**), β-sitosterol (**8**), β-sitosteryl palmitate (**9**), palmitic acid (**10**), 1-hydroxy-8-methoxyanthraquinone (**24**), and two other compounds were isolated.

Compound **30**, pale brown needles, was isolated from the fraction eluted by 15% ethyl acetate in Skellysolve B. The high resolution mass spectrum gives a molecular formula $C_9H_{10}O_3$. The compound possesses a hydroxyl (3140 cm$^{-1}$) and an aldehyde (2720 and 1705 cm$^{-1}$) group as shown by the ir spectrum. The nuclear magnetic resonance spectra indicate the presence of an aldehyde (δ 10.74, s, in $^1$H nmr and δ 191.77, s, in $^{13}$C nmr), hydroxyl (δ 4.27, br, $D_2O$ exchangeable, in $^1$H nmr and δ 165.68, s, in $^{13}$C nmr), methoxyl (δ 3.87, s, in $^1$H nmr and δ 56.27, q, in $^{13}$C nmr), and methyl (δ 2.55, s, in $^1$H nmr and δ 22.27, q, in $^{13}$C nmr) groups. The *meta* coupling constants (J = 2.5 Hz) of two aromatic hydrogens (δ 6.34, d and 6.24, d) in the $^1$H nmr spectrum (Figure 8) suggest a 1,2,3,5-tetrasubstituted benzene. In order to determine the substitutional pattern, difference nuclear Overhauser enhancement experiments were conducted. On irradiation of the methoxyl group, enhancement of an aromatic hydrogen signal (δ 6.34) is observed. On irradiation of the methyl group, enhancements of the other aromatic hydrogen (δ 6.24) and the aldehyde hydrogen signals are observed (Table 13). This information indicates that compound **30** is 4-hydroxy-2-methoxy-6-methylbenzaldehyde.

Figurer 8. $^{1}H$ nmr spectrum of isoevernin aldehyde ($CD_3OD$, 400 MHz)



30

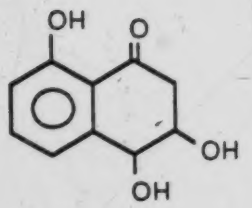
Table 13. The nOe data for compound 30

| Signal Irradiated | nOe (%) |
|---|---|
| H-3 6.43 | $OCH_3$ 3.87 (4.1) |
| $OCH_3$ 3.87 | H-3 6.34 (22.6) |
| H-5 6.24 | $CH_3$ 2.55 (2.5) |
| $CH_3$ 2.55 | CHO 10.47 (3.3) H-5 6.24 (15.9) |

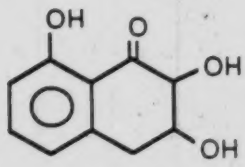
Compound **30**, named isoevernin aldehyde, has been previously isolated from the fungus *Guignardia laricina* [62]. The spectral properties of the metabolite **30** isolated from *V.* sp. C50 agree well with those reported for isoevernin aldehyde.

Compound **31**, which was crystallized from ethyl acetate as colorless needles, was obtained from the chromatographic fraction eluted with 30% ethyl acetate in Skellysolve B. It has a molecular formula $C_{10}H_{10}O_4$ as shown by the high resolution mass spectrum. The ir spectrum shows hydroxyl (3600-2400 $cm^{-1}$) and hydrogen-bonded carbonyl absorption (1650 $cm^{-1}$). The uv absorption maximum (259 nm) suggests an unsaturated ketone chromophore[63]. The $^1H$ nmr spectrum (Figure 9) in

methanol containing $D_2O$ exhibits three aromatic hydrogens, two carbinyl hydrogens (δ 4.62, d and 4.06, m), and two *geminal* methylene hydrogens (δ 3.09, dd and 2.69, dd). The aromatic hydrogens are present as an AMX system with *meta* and *ortho* coupling constants (J = 2.0, 8.0 Hz). This suggests the presence of 1,2,3-trisubstituted aromatic ring. Spin decoupling experiments show the coupling patterns of all hydrogen signals and reveal the presence of vicinal hydroxyl groups (Table 14). The $^{13}C$ nmr spectrum of compound 31 displays a ketone carbonyl (δ 204.36), two carbinyl methine carbons (δ 73.20, d and 71.57, d), one methylene carbon (δ 44.08, t), and six aromatic carbons. According to this spectral evidence, there are four possible structures (31-34) which may be considered for this compound.




31

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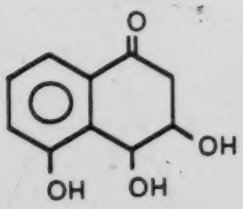
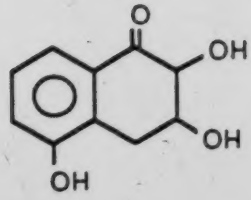
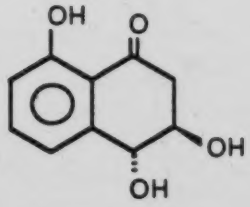



33




34

To distinguish between the structure possibilities, $^1H/^1H$ COSY and difference nOe spectra were obtained. In the COSY spectrum (Figure 10 and 11), a cross peak between one carbinyl hydrogen (δ 4.62, d) and one aromatic hydrogen (δ 6.86, dd) is observed. On irradiation of one carbinyl hydrogen (δ 4.62, d), enhancement of the aromatic hydrogen signal (δ 6.86, dd) is observed, and *vice versa* (Table 15). These data indicate that this compound is 3,4-dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone as depicted in structure **31**. Compound **31** is optically active ($[\alpha]_D$ -40°). The relative stereochemistry of the diol moiety was determined as follows. The coupling constants (J = 7.5 Hz) between the two carbinyl hydrogens suggests that these hydrogens are approaching diaxial[64,65]. In addition, no nOe enhancement between the two carbinyl hydrogens is observed and this observation is consistent with a trans diequatorial configuration for the vicinal hydroxyl groups. Thus compound **31** has the stereochemistry shown in **31a** or its enantiomer. Compound **31** has been previously isolated from the fungus *Pyricularia oryzae* Cavara[66]. The spectral properties (ir, $^1H$ nmr, ms, $[\alpha]_D$) of compound **31** are identical with those reported. Compound **31** is reported to be a phytotoxic substance, since it reduces the growth of rice seedlings when applied in high concentrations[66].




**31a**

Table 14. The spin decoupling $^1H$ nmr data for compound 31

| Signal | Irradiated | Observed Change |
|---|---|---|
| H-6 | 7.55 | 7.14 dd---d (2.0 Hz), 6.86 dd---d (2.0 Hz) |
| H-7 | 7.14 | 7.55 t----d (8.0 Hz), 6.86 dd---d (8.0 Hz) |
| H-5 | 6.86 | 7.55 t----d (8.0 Hz), 7.14 dd---d (8.0 Hz) |
| H-4 | 4.62 | 4.06 m---dd (7.5, 4.0 Hz) |
| H-3 | 4.06 | 4.62 d----s, 3.09 dd---d (17.5 Hz) |
| | | 2.69 dd---d (17.5 Hz) |
| H-2e | 3.09 | 4.06 m---t (7.5 Hz), 2.69 dd---d (7.5 Hz) |
| H-2a | 2.69 | 4.06 m---dd (7.5, 4.0 Hz) |
| | | 3.09 dd---d (4.0 Hz) |

Table 15. The nOe data for compound 31

| Signal | Irradiated | nOe (%) |
|---|---|---|
| H-5 | 7.14 | H-6 7.55 (7.0 ), H-4 4.62 (3.7) |
| H-4 | 4.62 | H-5 7.14 (5.7) |
| H-3 | 4.06 | H-2e 3.09 (2.5) |
| H-2e | 3.09 | H-3 4.06 (11.1) |

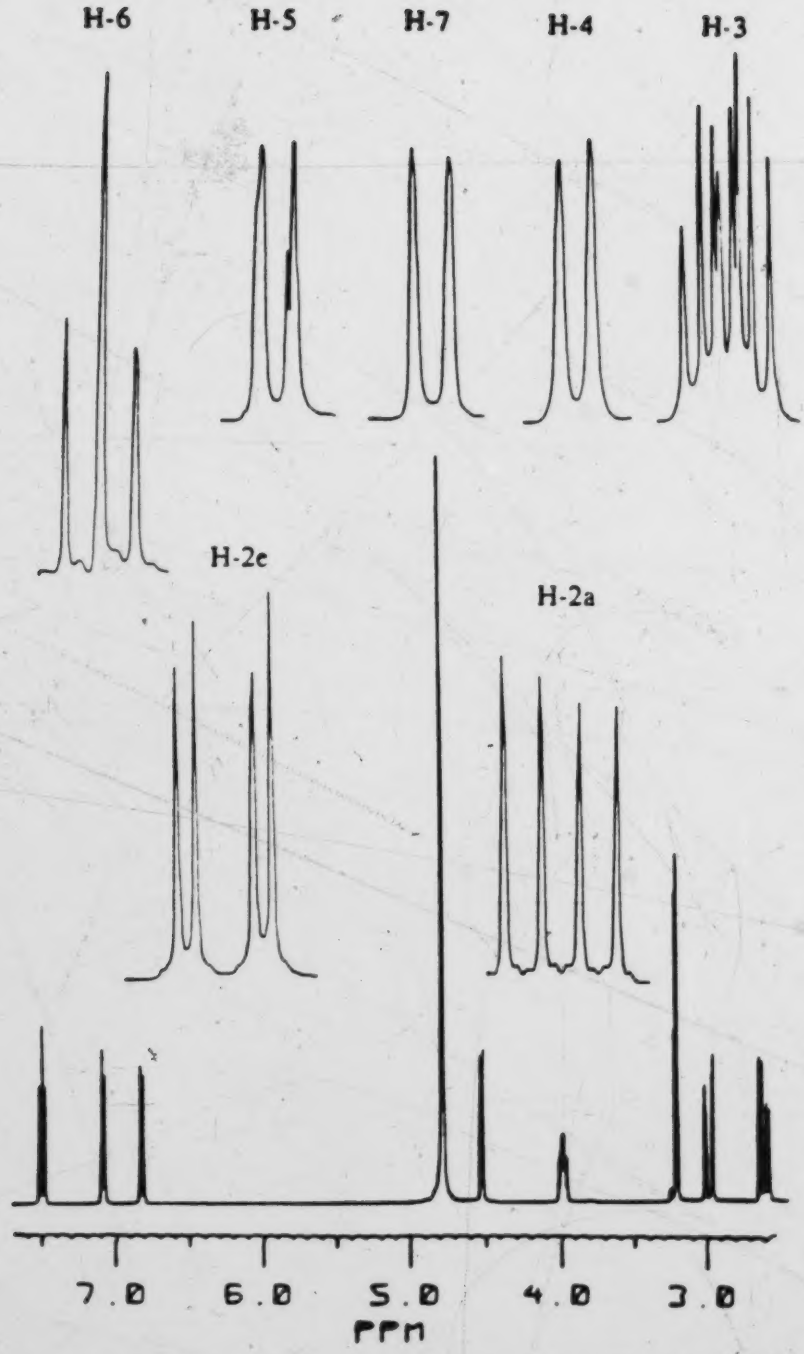



Figure 9. The $^1H$ nmr spectrum of compound 31 ($CD_3OD$, 360 MHz)

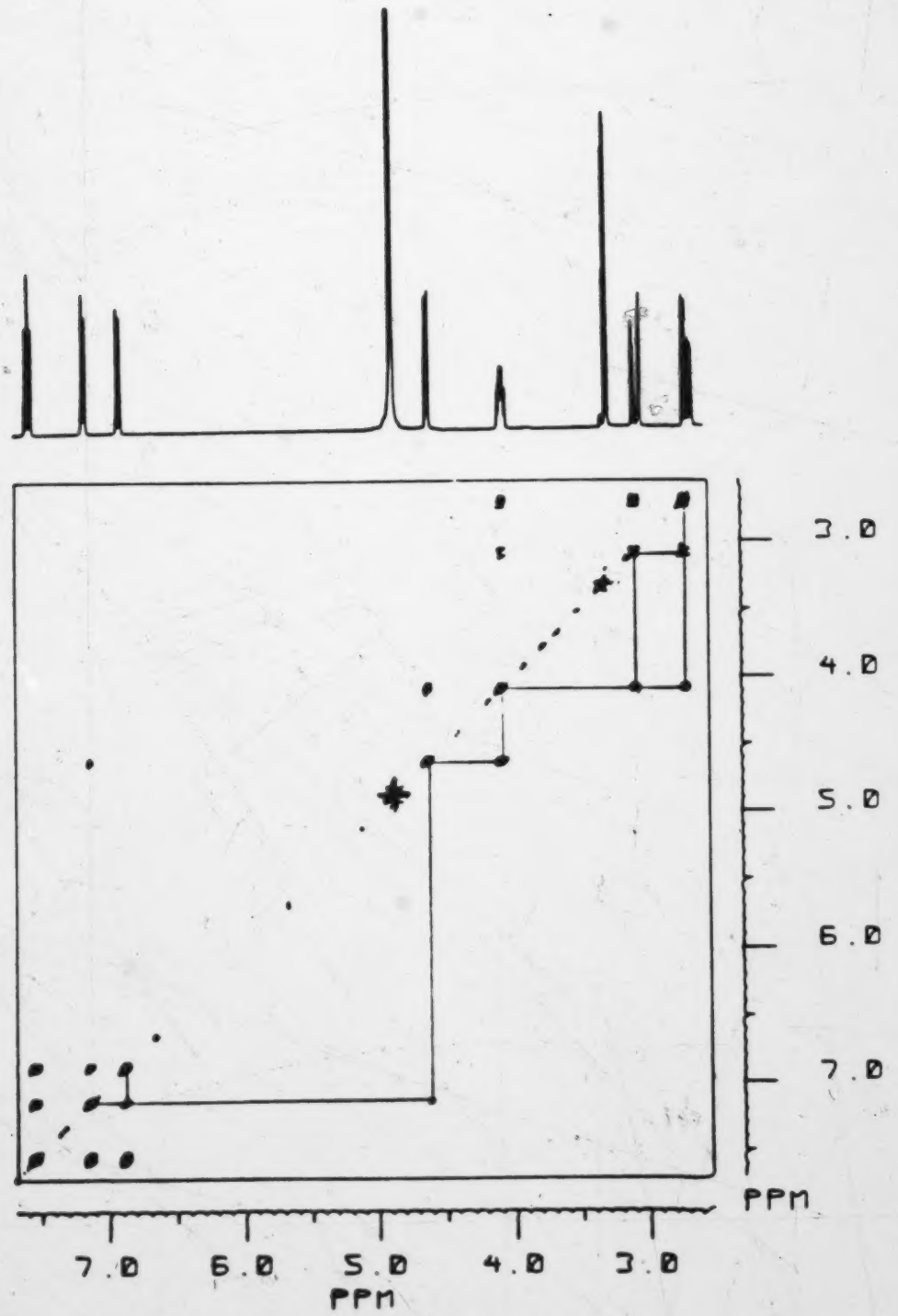



Figure 10. The $^1H/^1H$ COSY spectrum of compound 31 ($CD_3OD$, 360 MHz, COSY 90, contour plot)

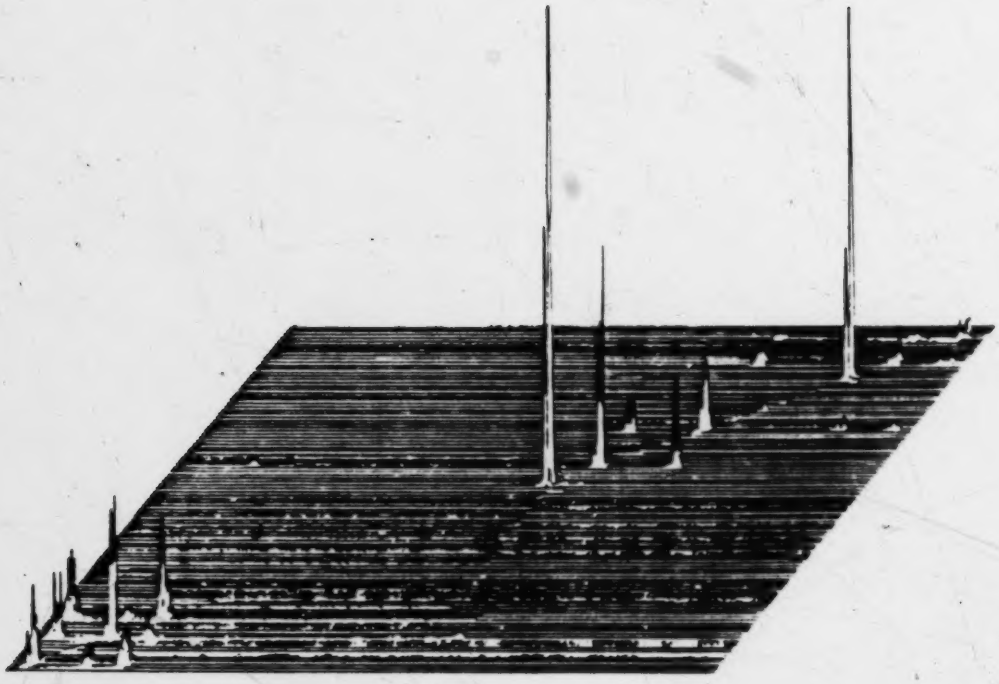


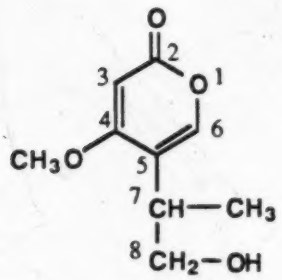
Figure 11. The $^{1}H/^{1}H$ COSY spectrum of compound **31**
($CD_3OD$, 360 MHz, COSY 90, stacked plot)

## 1.5 Metabolites of the ethyl acetate extract

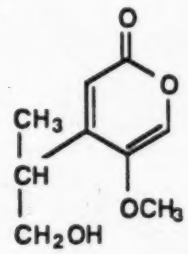
The crude ethyl acetate extract contains nine components as revealed by tlc. Silica gel flash chromatography led to the separation of seven components which were identified as 1,3,6,8-tetrahydroxyanthraquinone (3), 1,8-dimethoxynaphthalene (6), β-sitosterol (8), β-sitosteryl palmitate (9), vertixanthone (11), 1-hydroxy-8-methoxy-anthraquinone (24), and 2(3H)-benzoxazolone (29). In addition two new α-pyrone metabolites were isolated by charcoal column chromatography followed by silica gel flash chromatography.

Compound **35**, a new α-pyrone metabolite for which we propose the name vertipyronol was isolated as colorless oil. Its structure was derived from the chemical and spectral evidence presented below. Vertipyronol has a molecular formula $C_9H_{12}O_4$ as deduced by high resolution mass spectrum. Its ultraviolet spectrum indicates an α-pyrone chromophore (280 nm)[67], while its infrared spectrum shows hydroxyl (3417 $cm^{-1}$, br) as well as an α-pyrone carbonyl (1699 $cm^{-1}$, br) absorption[68,69]. The $^1H$ nmr spectrum of compound **35** (Table 20) displays an hydroxyl (δ 2.15, br, $D_2O$ exchangeable), a methoxyl (δ 3.90, s) and two olefinic hydrogens as singlets (δ 7.24 and 5.51). In addition there is a methine hydrogen (δ 2.95) which is coupled to methyl (δ 1.26) and methylene (δ 3.75) hydrogens. The coupling patterns for the hydrogens were verified by spin decoupling experiments (Table 16). In order to determine the position of the hydroxyl substitution, vertipyronol was acetylated with acetic anhydride in pyridine for 12 hours. The acetyl derivative, compound **37**, has a molecular formula $C_{11}H_{14}O_5$ (hrms). Its ir spectrum shows ester carbonyl (1745 $cm^{-1}$) and α-pyrone carbonyl absorption but no hydroxyl absorption, while its $^1H$ nmr spectrum shows one acetyl methyl signal (δ 2.04, s). In the $^1H$ nmr spectrum of acetylvertipyronol, the signals of the methylene hydrogens (δ 4.15 and 4.08) are shifted downfield by about 0.40 ppm relative to the same signals in

the $^{1}H$ nmr spectrum of vertipyronol . This information suggests that vertipyronol possesses a primary alcohol group, and thus the two substituents on the pyrone ring in vertipyronol must be a methoxyl and a hydroxyisopropyl group. In the $^{1}H$ nmr spectrum of vertipyronol, the two olefinic hydrogens are assigned to the 3 (δ 5.51) and 6 (δ 7.24) positions, based on comparison to the chemical shift of the analogous hydrogens of α-pyrones[70,71]. Thus vertipyronol is a 4,5-disubstituted α-pyrone. At this point, two possible structures (**35**, **36**) may be considered for vertipyronol.




**35**




**36**

Difference nOe experiments were used to distinguish between the two possible structures. Upon irradiation of H-6, enhancements of the methyl and the methine signal are observed. Upon irradiation of the methoxyl signal, enhancement of H-3 is observed, and *vice versa* (Table 17). This evidence is consistent with structure **35** for that of vertipyronol. The normal and fully coupled $^{13}C$ nmr spectra of vertipyronol exhibit carbon signals (Table 21) consistent with the assigned structure[72]. The fragmentation patterns of vertipyronol (**35**) (Scheme 7) and the monoacetyl derivative **37** (Scheme 8) shown in the high resolution mass spectra are also in agreement with the assigned structure[73]. Vertipyronol is optically active ($[\alpha]_D$ -1.58°), however, the

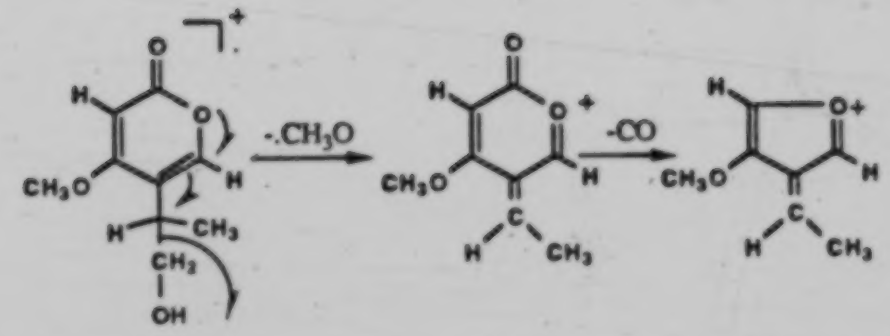
absolute confinguration at C-7 has not been determined. Some naturally occurring α-pyrone metabolites have been reported to have antibiotic activity[74-76].

Table 16. The spin decoupling $^1H$ nmr data for vertipyronol

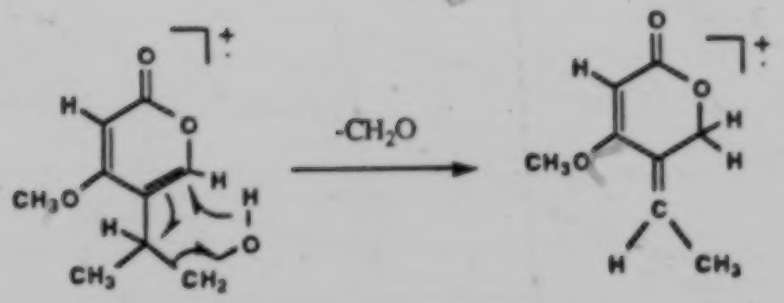
| Signal Irradiated | Observed Change |
|---|---|
| 3.75 3.65 2x H-8 | 2.95 H-7 tq---q (7.5 Hz) |
| 2.95 H-7 | 3.75 H-8 dd---d (10.5 Hz) |
| | 3.65 H-8 dd---d (10.5 Hz) |
| | 1.26 $CH_3$ d----s |
| 1.26 $CH_3$ | 2.95 H-7 tq---t (6.0 Hz) |

Table 17. The nOe data for vertipyronol

| Signal Irradiated | nOe (%) | |
|---|---|---|
| 7.24 H-6 | 2.95 H-7 (3.7) | 1.26 $CH_3$ (1.6) |
| 5.51 H-3 | 3.90 $OCH_3$ (3.1) | |
| 3.90 $OCH_3$ | 5.51 H-3 (16.9) | |
| 3.75 H-8 | 3.65 H-8 (4.4) | 2.95 H-7 (3.5) |
| 3.65 H-8 | 3.75 H-8 (3.2) | 2.95 H-7 (5.6) |
| 2.95 H-7 | 7.24 H-6 (3.2) | 3.75 H-8 (0.9) |
| | 3.65 H-8 (1.1) | 1.26 $CH_3$ (1.4) |
| 1.26 $CH_3$ | 7.24 H-6 (12.9) | 2.95 H-7 (12.9) |

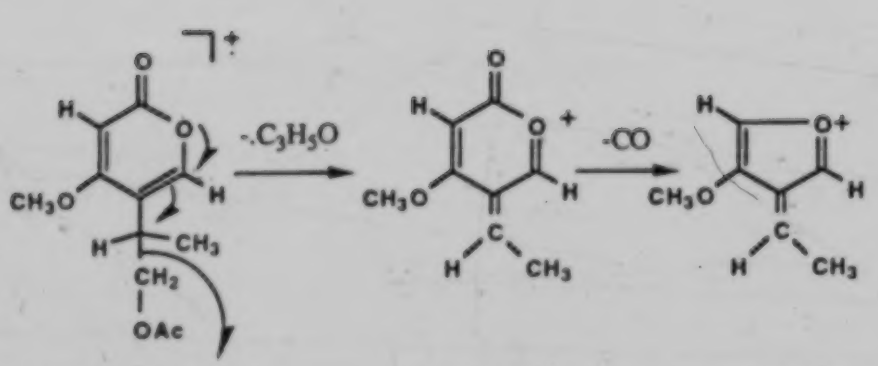


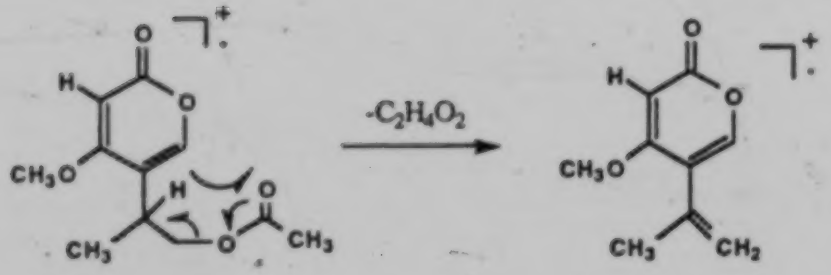

M⁺ m/z 184 (18) m/z 153 (100) m/z 125 (6)




M⁺ m/z 184 (18) m/z 154 (11)

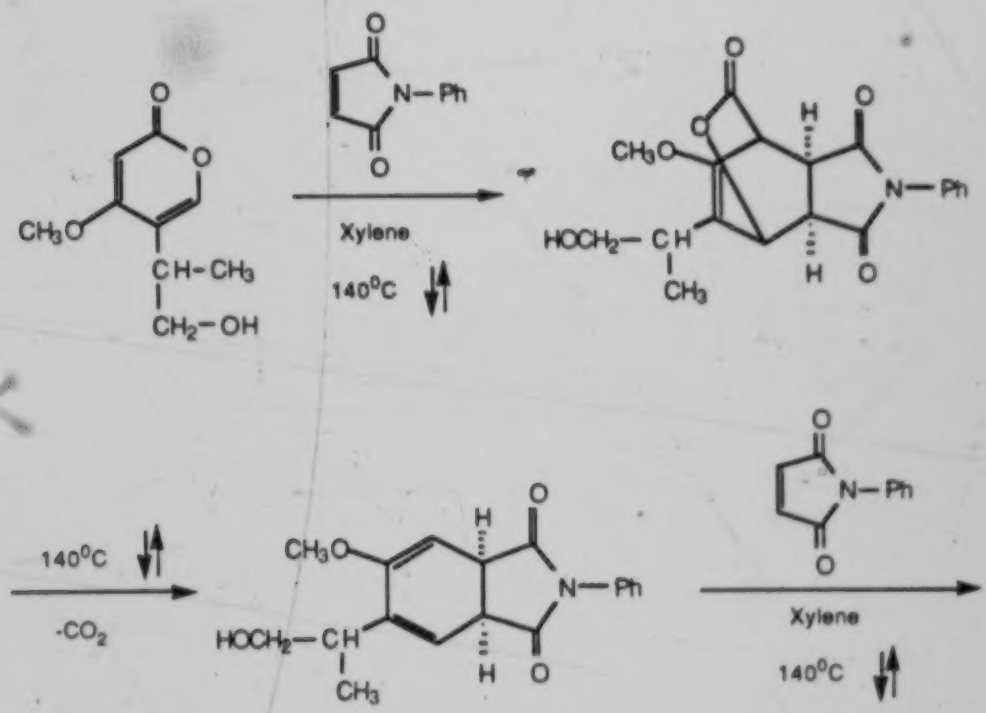
Scheme 7. The fragmentation of vertipyronol

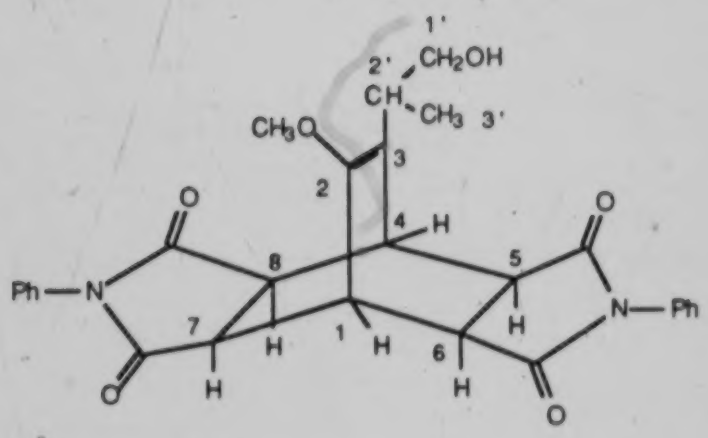



Scheme 8. The fragmentation of compound **37**

To further confirm the proposed structure, vertipyronol (**35**) was treated with N-phenylmaleimide in refluxing toluene to give compound **38** (Scheme 9).



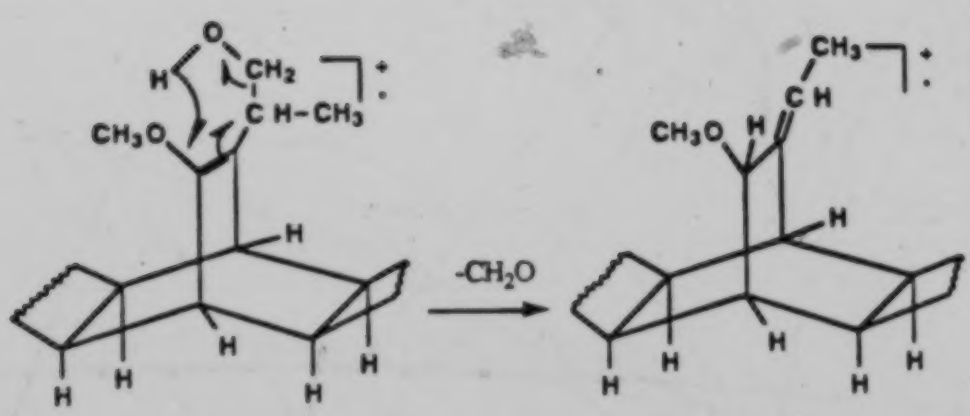
38

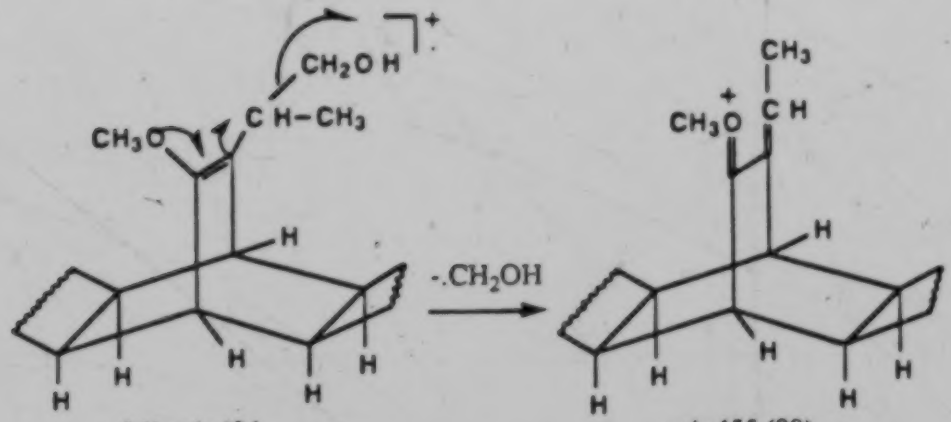
Scheme 9. Diels-Alder reaction of vertipyronol

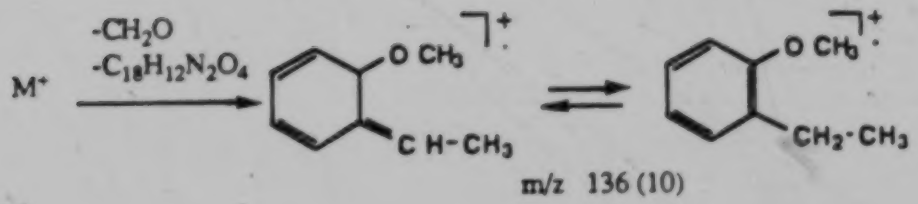
The adduct, compound **38**, has a molecular formula $C_{28}H_{26}N_2O_6$ as shown by high resolution mass spectrum. It is optically active ($[\alpha]_D$ -3.06°). The infrared spectrum shows imide absorption (1715 and 1499 $cm^{-1}$). The $^1H$ nmr spectrum (Figure 12) is complicated, however, the hydrogen signals were resolved by spin decoupling (Table 18) and assigned by $^1H/^1H$ COSY experiments (Figure 13-15). The cross peaks in the COSY spectrum indicate that H-1 (δ 4.25, t) is coupled to H-6 and H-7 (δ 3.23, m), while H-4 (δ 3.91, t) is coupled to H-5 and H-8 (δ 3.14, dd). The coupling constants between H-5 and H-6 (J = 8.0 Hz) and between H-1 and H-6 (H-7) (J = 3.0 Hz) indicate an *endo -endo* arrangement[77-80]. Therefore, the stereochemistry of the adduct is assigned as the *exo -exo* . The fragmentation of compound **38** in the mass spectrum as outlined in Scheme 10 is consistent with the assigned structure .

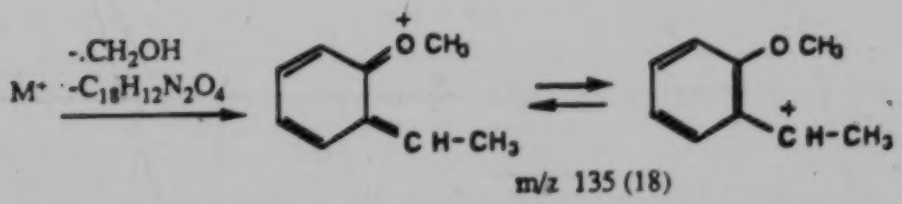
Table 18. The spin decoupling $^1H$ nmr data for compound **38**

| Signal Irradiated | Observed Change |
|---|---|
| 4.25 H-1 | 3.23 H-6 H-7 m---2xd (8.0 Hz) |
| 3.91 H-4 | 3.14 H-5 H-8 2xdd---2xd (8.0 Hz) |
| 3.40 H-1' | 2.73 H-2' tq---q (7.0 Hz) |
| 3.23 H-6 H-7 | 4.25 H-1 t---s |
| | 3.14 H-5 H-8 2xdd---2xd (3.0 Hz) |
| 3.14 H-5 H-8 | 3.91 H-4 t---s |
| | 3.23 H-6 H-7 m---2xd (3.0 Hz) |
| 2.73 H-2' | 3.40 H-1' t---d (6.5 Hz) |
| | 0.86 H-3' d---s |
| 0.86 H-3' | 2.73 H-2' tq---t (6.5 Hz) |



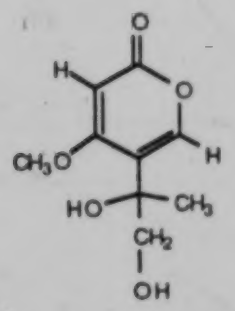




Scheme 10. The fragmentation of compound **38**

Compound 39, a colorless crystalline compound, was isolated by chromatography over charcoal and then silica gel. It is a new α-pyrone metabolite which we have named vertipyronediol. Its structure was determined by spectral studies and by comparison of its spectral properties with those of vertipyronol (35). Vertipyronediol (39) has a molecular formula $C_9H_{12}O_5$ (hrms m/z: $M^+$, 200; cims m/z: $M^+ + 1$, 201, $M^+ + 18$, 218), which differs from that of vertipyronol (35) by one oxygen atom. The uv spectrum indicates the α-pyrone chromophore (279 nm)[67]. The ir spectrum shows the presence of hydroxyl (3390, 3291 $cm^{-1}$) and an α-pyrone carbonyl (1711 $cm^{-1}$) absorption. The $^1H$ nmr spectrum of vertipyronediol (39) (Table 20) is similar to that of vertipyronol (35) except that in the spectrum of compound 39 there are two hydroxyl signals (δ 3.18, br and 1.75, br, $D_2O$ exchangeable), and the methyl signal is a singlet while the methylene signals appear as doublets. This information suggests that vertipyronediol has the same skeleton as that of vertipyronol (35), and contains one more hydroxyl group. The spin patterns of the methyl and the methylene signals in the $^1H$ nmr spectrum of compound 39 suggest that the additional hydroxyl group is located at C-7. Hydroxyl substitution at C-7 is further supported by the $^{13}C$ nmr spectrum of vertipyronediol (39), since the chemical shift of C-7 (δ 73.67, s) is downfield relative to that of vertipyronol (35) (δ 32.84, d). The fully coupled $^{13}C$ nmr spectrum of vertipyronediol (Table 21) shows that the multiplicity of C-6, the methyl, and the methylene carbon differ from those of vertipyronol (35), since there is no long range C-H coupling between H-7 and these carbons. This evidence further confirms the hydroxyl substitution and shows that vertipyronediol (39) contains a 2-(1,2-propanediol) group. Treatment of vertipyronediol (39) with acetic anhydride in pyridine at room temperature for 12 hours gave an acetyl derivative, compound 40. The reaction product has a molecular formula $C_{11}H_{14}O_6$ (hrms). The ir spectrum shows an α-pyrone carbonyl, hydroxyl absorption and an ester carbonyl absorption (1739 $cm^{-1}$). The $^1H$ nmr spectrum

displays one acetyl methyl signal (δ 2.06, s), and the methylene hydrogen signals shift downfield about 0.40 ppm relative to the same signals in the $^1H$ nmr spectrum of the unacetylated compound (Table 20). This shows that the reaction product **40** is a monoacetyl derivative of vertipyronediol (**39**) and that acetylation took place at the primary alcohol. This provides further evidence that vertipyronediol (**39**) has a primary hydroxyl group at C-8 and a tertiary hydroxyl group at C-7.

Difference nOe experiments (Table 19) were used to verify the position of the substituents of vertipyronediol. On irradiation of the C-methyl signal, enhancements of H-6 and the methylene signals are observed. On irradiation of the methoxyl signal, enhancement of H-3 is observed, and *vice versa*. Therefore, vertipyronediol is identified as **39**.



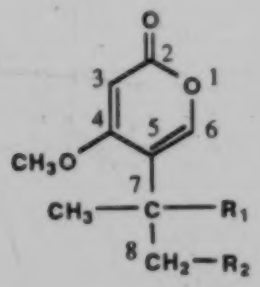

**39**

The mass spectral fragmentation of vertipyronediol (**39**) (Scheme 11) and its acetyl derivative **40** (Scheme 12) is in good agreement with the proposed structure. Vertipyronediol is optically active ($[\alpha]_D$ -5.0°). However, the absolute configuration at C-7 has not been determined. Biogenetically, the α-pyrone metabolites produced by

*Verticicladiella* may be derived from a tetraketide intermediate, and by way of an oxidative cleavage of an orsellinic acid derivative[81]. It is of interest to note that in all known naturally occurring methoxy-α-pyrones, the methoxyl is located on the 4 position[82-86].

Table 19. The nOe data for vertipyronediol

| Signal Irradiated | nOe (%) |
|---|---|
| 7.65 H-6 | 1.52 $CH_3$ (1.4) |
| 5.56 H-3 | 3.90 $OCH_3$ (3.3) |
| 3.90 $OCH_3$ | 5.56 H-3 (22.2) |
| 3.98 H-8 | 3.64 H-8 (27.9), 1.52 $CH_3$ (0.8) |
| 3.64 H-8 | 3.98 H-8 (23.3), 1.52 $CH_3$ (1.4) |
| 1.52 $CH_3$ | 7.65 H-6 (4.9), 3.98 H-8 (1.2), 3.64 H-8 (2.4) |




| | $R_1$ | $R_2$ |
|---|---|---|
| **35** | H | OH |
| **37** | H | OAc |
| **39** | OH | OH |
| **40** | OH | OAc |

Table 20. The $^1H$ nmr data for α-pyrone compounds

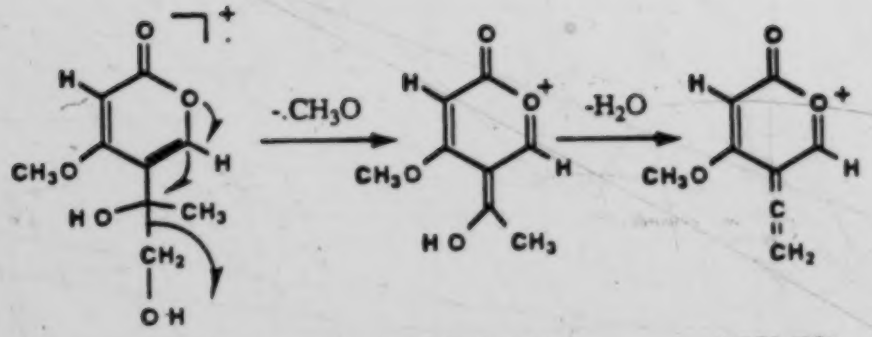
| | Chemical Shift (mult. J in Hz) ($CDCl_3$, 360 MHz) | | | |
|---|---|---|---|---|
| H | 35 | 37 | 39 | 40 |
| 3 | 5.51 (s) | 5.56 (s) | 5.56 (s) | 5.58 (s) |
| 6 | 7.24 (s) | 7.22 (s) | 7.65 (s) | 7.61 (s) |
| 8 | 3.75 (dd) | 4.15 (dd) | 3.98 (d) | 4.39 (d) |
| | (10.5,6.0) | (10.5,6.0) | (11.0) | (11.5) |
| 8 | 3.65 (dd) | 4.08 (dd) | 3.64 (d) | 4.27 (d) |
| | (10.5,6.0) | (10.5,6.0) | (11.0) | (11.5) |
| $CH_3$ | 1.26 (d) | 1.22 (d) | 1.52 (s) | 1.50 (s) |
| $OCH_3$ | 3.90 (s) | 3.85 (s) | 3.90 (s) | 3.88 (s) |
| R1 | 2.95 (tq) | 3.05 (tq) | 3.18 (br) | 3.01 (br) |
| | (6.0,7.5) | (6.0,7.5) | | |
| R2 | 2.15 (br) | 2.04 (s) | 1.75 (br) | 2.06 (s) |

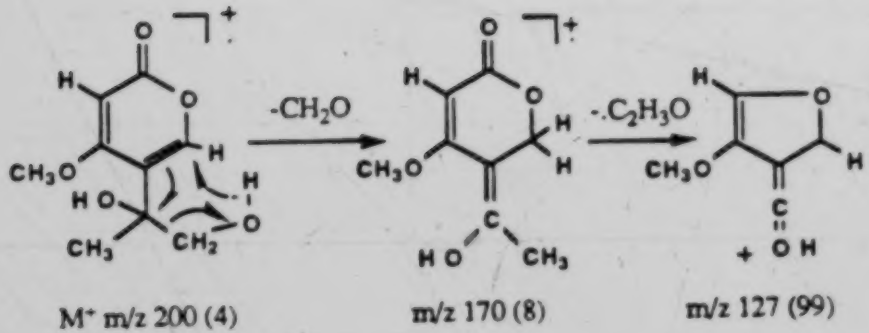
Table 21. The $^{13}C$ nmr data for vertipyronol and vertipyronediol

| | Chemical Shift (mult. J in Hz) (CDCl$_3$, 75 MHz) | | | |
|---|---|---|---|---|
| | 35 | | 39* | |
| C | APT | FC | APT | FC |
| 2 | 164.42(s) | (br) | 167.41(s) | (br) |
| 3 | 90.00(d) | (d, 169.8) | 90.55(d) | (d, 176.81) |
| 4 | 169.97(s) | (d, 7.0) | 171.64(s) | (d, 8.0) |
| 5 | 117.08(s) | (br) | 120.89(s) | (br) |
| 6 | 148.65(d) | (dd, 197.0, 7.0) | 151.86(d) | (d, 203.5) |
| 7 | 32.84(d) | (d.br, 128.9) | 73.67(s) | (br) |
| 8 | 65.75(t) | (t,quintet, 143.0, 5.0) | 69.16(t) | (tq, 140.1, 4.0) |
| $\underline{C}H_3$ | 15.85(q) | (qq, 127.1, 3.1) | 24.50(q) | (q, 128.0) |
| $O\underline{C}H_3$ | 56.08(q) | (q, 146.6) | 56.65(q) | (q, 146.0) |

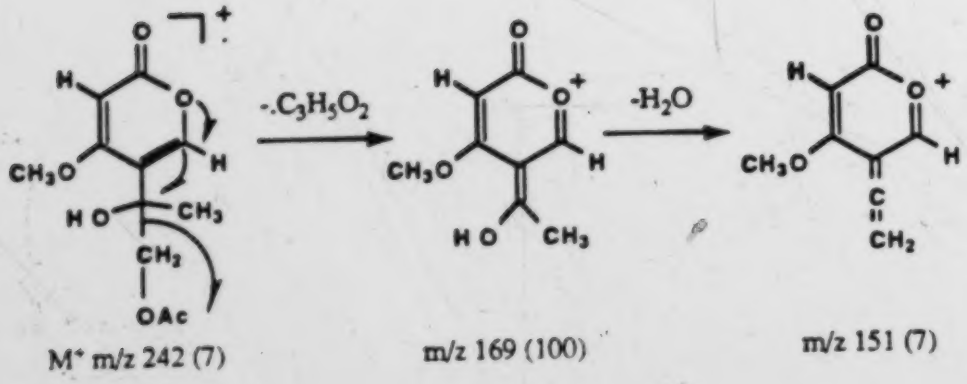
* In $CD_3OD$.

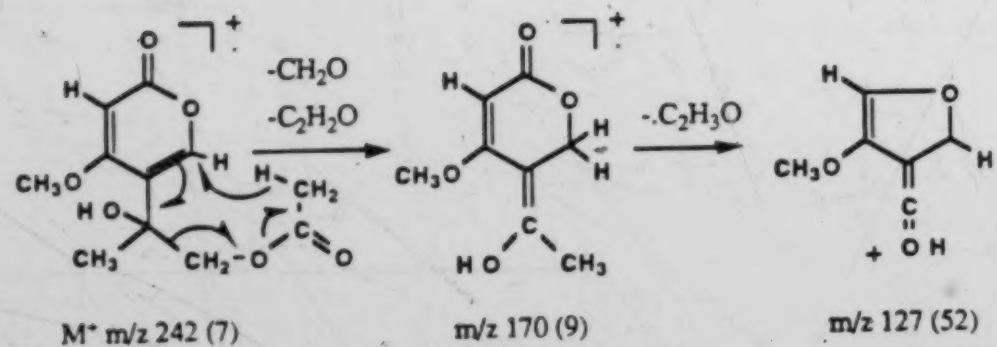
APT: Attached Pronton Test, FC: Fully Coupled.




Scheme 11. The fragmentation of vertipyronediol




Scheme 12. The fragmentation of compound **40**

Figure 12. The $^{1}H$ nmr spectrum of compound 38 ($CDCl_3$, 360MHz)

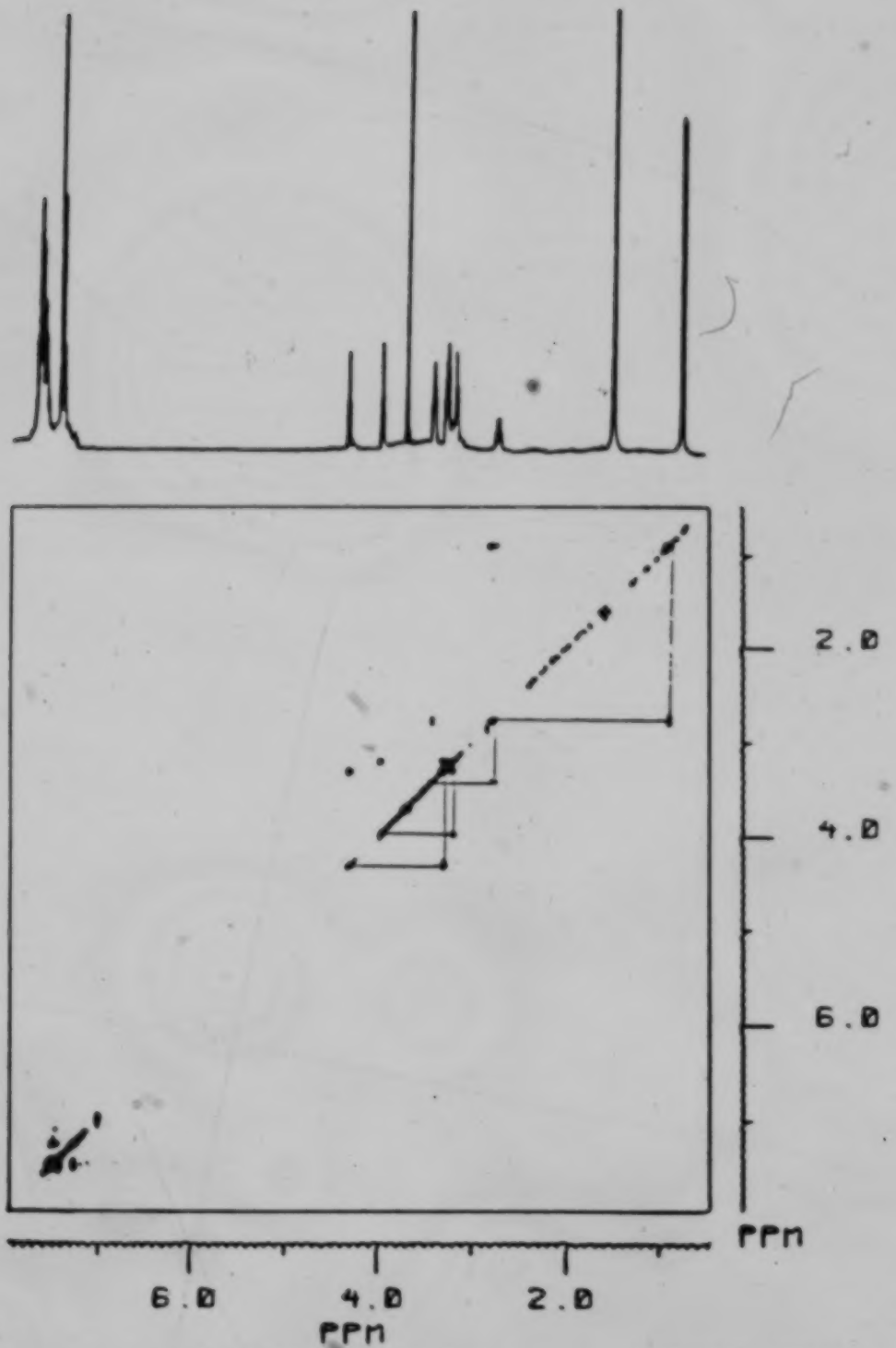



Figure 13. The $^1H/^1H$ COSY spectrum of compound 38 ($CDCl_3$, 360 MHz, COSY 90, contour plot)

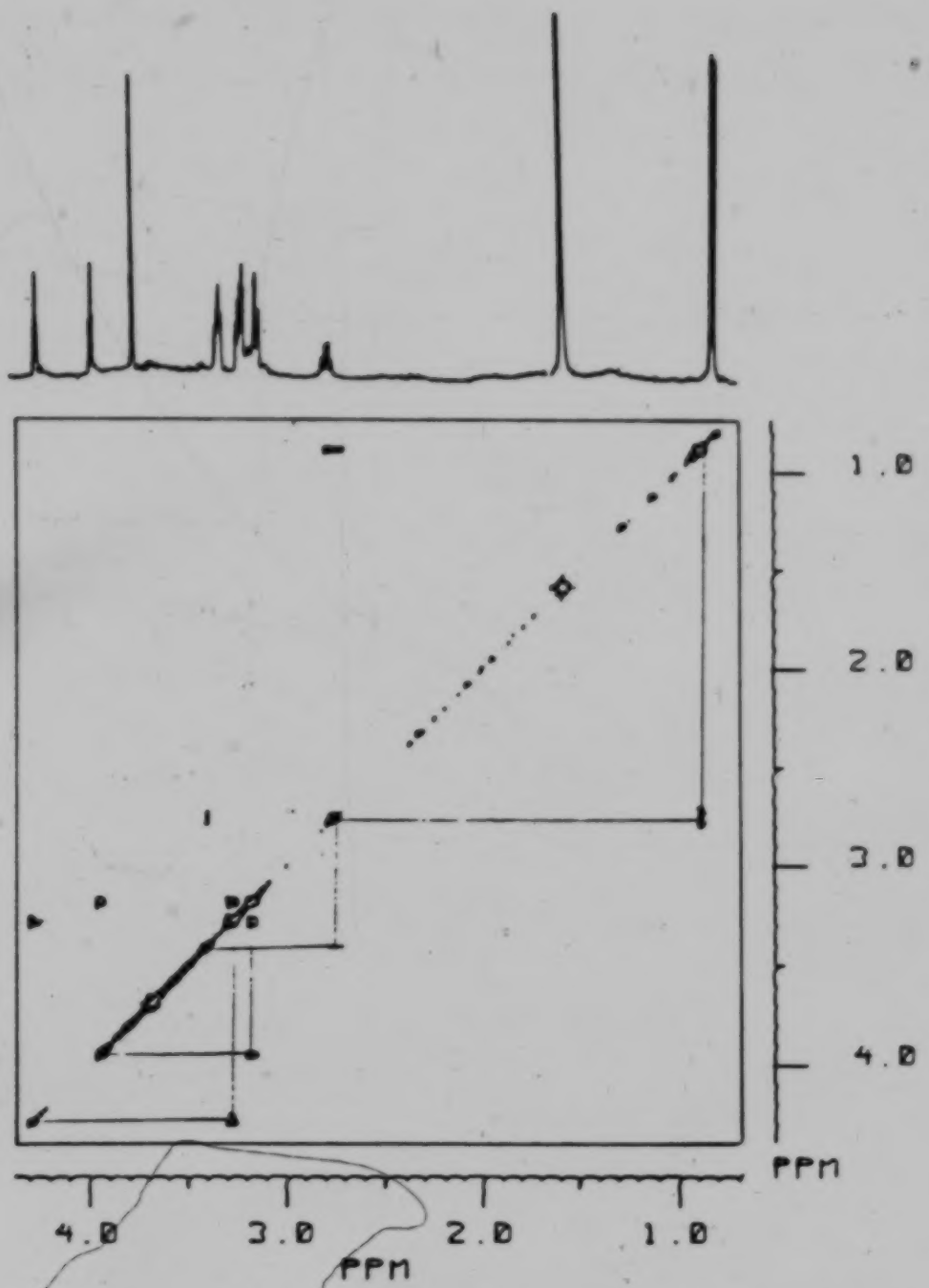



Figure 14. The $^1H/^1H$ COSY spectrum of compound 38 ($CDCl_3$, 360 MHz, COSY 90, contour plot, expansion)

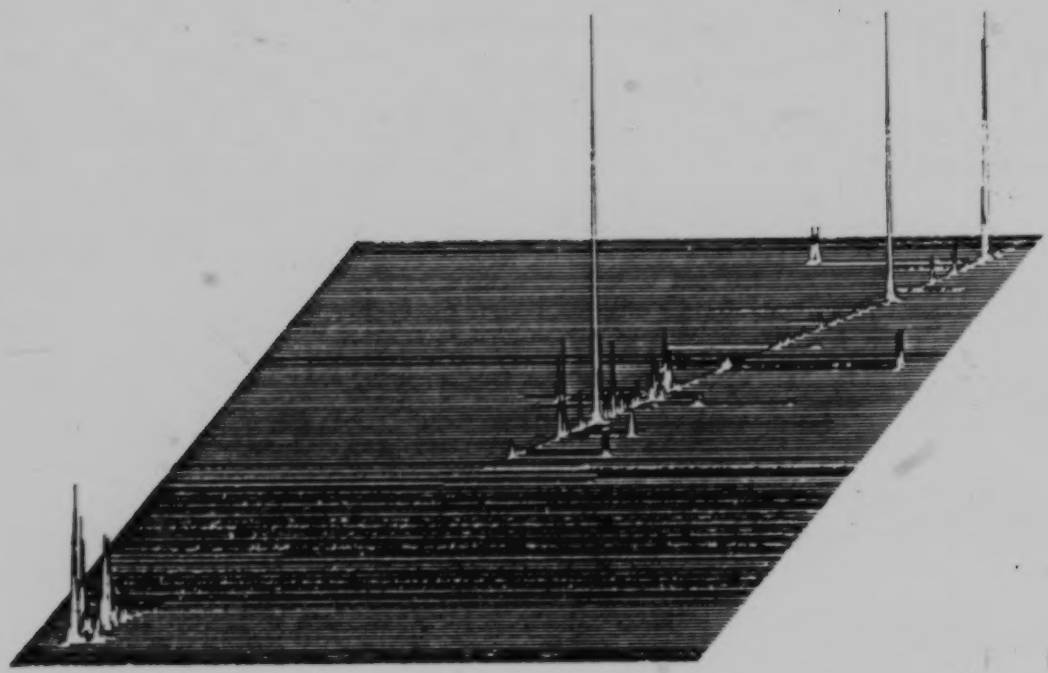


Figure 15. The $^1H/^1H$ COSY spectrum of compound **38** ($CDCl_3$, 360 MHz, COSY 90, stacked plot)

## 2. Metabolites of *Verticicladiella* sp. C728 Grown on Solid Medium

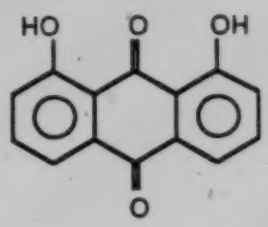
*Verticicladiella* sp. C728 was grown on solid rye medium, in a manner similar to that described for *Verticicladiella* sp. C50. The solid culture was extracted with Skellysolve B, ether, methylene chloride, and ethyl acetate to give four crude extracts. The metabolites of each extract were separated by chromatography. Nine compounds have been isolated, seven of which were also obtained from strain C50. The seven previously identified metabolites are 1,3,6,8-tetrahydroxyanthraquinone (**3**), 1,8-dimethoxynaphthalene (**6**), β-sitosteryl palmitate (**9**), palmitic acid (**10**), vertixanthone (**11**), 1-hydroxy-8-methoxyanthraquinone (**24**), and 5-*n*-nonadecylresorinol (**28**). The pure compounds isolated from each crude extract are listed in Table 22.

Table 22. Metabolites of four extracts of C728 grown on solid medium

| Extract | Compound | | | | | |
|---|---|---|---|---|---|---|
| SKB | 6 | 11 | 24 | 41 | 42 | |
| $Et_2O$ | 3 | 11 | 24 | 28 | 41 | 42 |
| $CH_2Cl_2$ | 9 | 10 | 42 | | | |
| EtOAc | 3 | 9 | 10 | 41 | | |

Compound **41** was isolated as yellow needles by silica gel flash chromatography eluting with 5% ethyl acetate in Skellysolve B. It turns red when dissolved in basic solution. The ultraviolet spectrum of compound **41** indicates an α-hydroxyanthraquinone chromophore (Table 9). It has a molecular formula $C_{14}H_8O_4$.

which differs from that of 1-hydroxy-8-methoxyanthraquinone (**24**) by a $CH_2$ unit. The infrared spectrum of pigment **41** shows two carbonyl (1628 and 1664 $cm^{-1}$) and hydroxyl (3100 $cm^{-1}$, br) absorptions. The $^1H$ nmr spectrum is similar to that of compound **24**, except that there are two hydroxyl hydrogens (δ 12.08, 2H, s, $D_2O$ exchangeable) and no methoxyl signal for pigment **41**. The six aromatic hydrogens are displayed as two separate AMX systems with *ortho* and *meta* coupling constants (Table 10) indicating the presence of two 1,2,3-trisubstituted aromatic rings. The $^{13}C$ nmr spectrum of compound **41** (Table 11) displays two carbonyl carbon signals (δ 193.16 and 181.77) suggesting 1,8-disubstitution from symmetry considerations. This yellow pigment is identified as 1,8-dihydroxyanthraquinone (**41**). Previously it was known as a synthetic compound, and recently it has been isolated from the coral *Tubastraea micrantha* Ehrenberg [87]. However, we believe this is the first time it has been isolated from fungul sources. All spectral data of compound **41** are identical with reference values[88-91]. Previous biological studies[92] have shown that 1,8-dihydroxyanthraquinone has antibiotic activity, especially against *Gram* positive bacteria. Antibiotic bioassay tests in our laboratories show similar results (see section 5, Biological Studies).




**41**

The thin layer chomatography behaviour of the three anthraquinone compounds are unusual. The 1,8-dihydroxyanthraquinone (41) gives the highest $R_f$ value, while 1,8-dimethoxyanthraquinone (26) appears at the bottum on the tlc plate (Figure 16). This observation is in agreement with that reported in the literature[93] and may arise because the hydrogens on hydroxyl groups can be hydrogen bonded to the carbonyl function. Thus the $R_f$ order (Table 23) is due to the availability of methoxyl and carbonyl groups to interact with the silica gel or alumina.

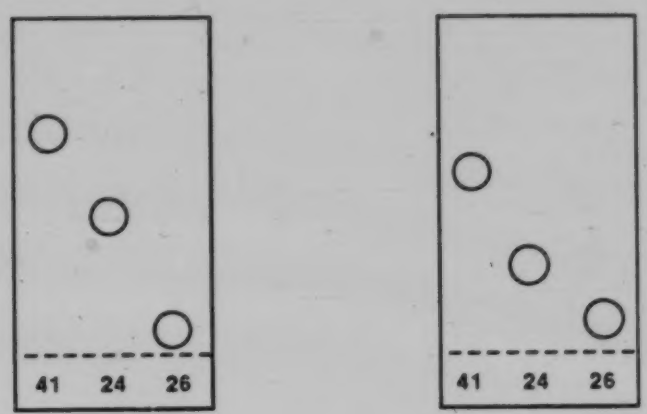


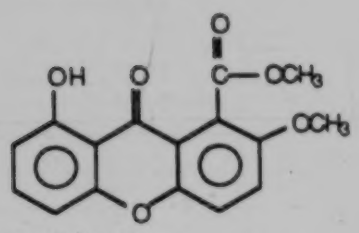

Figure 16. The tlc characteristics of anthraquinone compounds

Table 23. The $R_f$ values of anthraquinone compounds

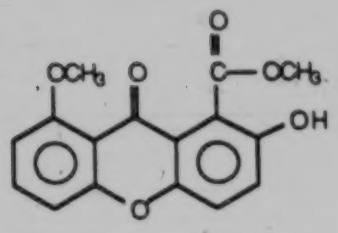
| Compound | $SiO_2$ | $Al_2O_3$ |
|---|---|---|
| 24 | 0.40 | 0.29 |
| 26 | 0.07 | 0.09 |
| 41 | 0.66 | 0.59 |

Solvent: $CHCl_3$

A yellow crystalline pigment, compound 42, was isolated from the chromatographic fraction eluted with 30% ethyl acetate in Skellysolve B. It is a xanthone as shown by the uv spectrum (Table 6). The high resolution mass spectrum shows a molecular formula $C_{16}H_{12}O_6$, which differs from that of hydroxyvertixanthone (16) by a $CH_2$ unit. The ir spectrum exhibits hydroxyl (3300 $cm^{-1}$), ester carbonyl (1756 $cm^{-1}$), and conjugated carbonyl (1651 $cm^{-1}$) absorptions. The $^1H$ nmr spectrum of pigment 42 is similar to that of compound 16 (Table 7) except that the former displays one hydroxyl signal (δ 12.25, s, $D_2O$ exchangeable) and two methoxyl signals (δ 3.92, s and 4.05, s). The five aromatic hydrogens are displayed as an AB and an AMX spin system with *ortho* and *meta* coupling constants. This suggests the presence of one 1,2,3-trisubstituted and one 1,2,3,4-tetrasubstituted aromatic rings. The $^{13}C$ nmr spectrum (Table 8) of pigment 42 and compound 16 are similar except that compound 42 has one more methoxyl carbon signal (δ 57.05, q). This indicates that compound 42 has the same substituted xanthone skeleton as that of hydroxyvertixanthone (16). Therefore, this pigment must be either structure 42 or 43.



42



43

In order to distinguish between the two possible structures, difference nOe experiments were carried out. On irradiation of the methoxyl signal, enhancement of the B hydrogen of the AB system and *vice versa* is observed (Table 24), indicating methoxyl substitution at C-2. Thus this pigment is methyl 2-methoxyl-8-hydroxy-xanthone-1-carboxylate (**42**). This compound has been previously isolated from the fungus *Mycosphaerella rosigena* and named mycoxanthone[94]. Comparison of the spectral properties of compound **42** (uv, ir, $^1$H nmr, ms) with those reported for mycoxanthone confirms the identity.

Table 24. The nOe data for mycoxanthone

| Signal Irradiated | nOe (%) |
|---|---|
| 7.42 H-B | 3.92 $OCH_3$ (4.7) |
| 3.92 $OCH_3$ | 7.42 H-B (24.2) |

## 3. Metabolites of *Verticicladiella* sp. C50 Grown in Liquid Medium

### 3.1 Isolation of the crude extracts

*Verticicladiella* sp. C50 was grown in liquid still culture on 10% filtered V-8 juice containing 1% glucose. After six weeks the culture broth was decanted from the mycelium. The mycelium was subjected to successive continuous extractions in a Soxhlet extractor with Skellysolve B and ethyl acetate. The culture broth was concentrated to small volume *in vacuo* and continuously extracted with Skellysolve B then ethyl acetate for 24 hours.

### 3.2 Metabolites from the mycelium extracts

The crude Skellysolve B extract contains one major component and some fatty acids. Chromatography of the crude extract led to the isolation of 1,8-dimethoxy-naphthalene (6).

The crude ethyl acetate extract was separated by silica gel flash chromatography using gradient elution with Skellysolve B and ethyl acetate. Five compounds identical with those isolated previously from strain C50 (solid medium, rye) have been obtained. The metabolites were identified as 1,3,6,8-tetrahydroxy-anthraquinone (3), 1,8-dimethoxynaphthalene (6), vertixanthone (11), hydroxy-vertixanthone (16), and 1-hydroxy-8-methoxyanthraquinone (24).

3.3 Metabolites from the culture broth extracts

1,8-Dimethoxynaphthalene (**6**) was isolated form the crude Skellysolve B extract. 1,8-Dimethoxynaphthalene (**6**), vertixanthone (**11**), 1-hydroxy-8-methoxy-anthraquinone (**24**), and 3,4-dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone (**31**) were obtained from the crude ethyl acetate extract by silica gel flash chromatography.

4. Comparison of Metabolites of *Verticicladiella* sp. C50 and C728 Grown on the Same or Different Media

Investigation of the metabolites from two strains C50 an C728 grown on solid medium (rye) indicates that these two strains produce similar components. In addition, a single strain, C50, when grown on different media, (solid (rye) and liquid (V-8 juice)), produces the same type of metabolites. However, larger quantities of metabolites are obtained when it is grown on the solid medium (rye). Comparisons of the metabolites isolated from the two strains when grown on the same medium and the single strain (C50) when grown on different media are shown in the Tables 25 and 26.

Table 25*. Metabolites of *Verticicladiella* sp. C50 and C728 grown on solid medium (rye)

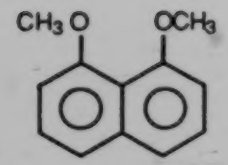
| Extract | C50 | C728 |
|---|---|---|
| SKB | 6, 8 | 6, 11, 24, 41, 42 |
| $Et_2O$ | 3, 6, 9, 11, 16, 24, 28, 29 | 3, 11, 24, 28, 41, 42 |
| $CH_2Cl_2$ | 6, 8, 9, 10, 24, 30, 31 | 9, 10, 42 |
| EtOAc | 3, 6, 8, 9, 11, 24, 29, 35, 39 | 3, 9, 10, 41 |

Table 26*. Metabolites of *Verticicladiella* sp. C50 grown on solid medium (rye) and liquid medium (V-8 juice)

| Solid Medium | | Liquid Medium | |
|---|---|---|---|
| SKB | 6, 8 | SKB (M) | 6 |
| $Et_2O$ | 3, 6, 9, 11, 16, 24, 28, 29 | EtOAc (M) | 3, 6, 11, 16, 24 |
| $CH_2Cl_2$ | 6, 8, 9, 10, 24, 30, 31 | SKB (B) | 6 |
| EtOAc | 3, 6, 8, 9, 11, 24, 29, 35, 39 | EtOAc (B) | 6, 16, 24, 31 |

M: Mycelium extract; B: Broth extract.

* The structure of the various compounds are shown on the next page.



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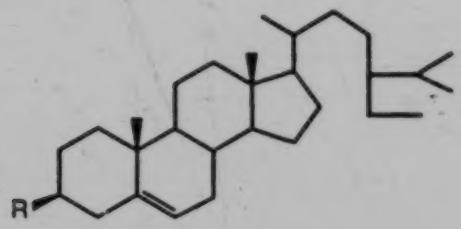
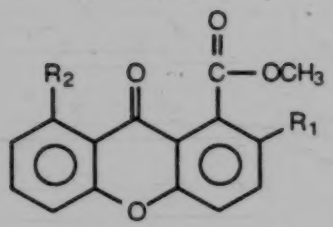


R

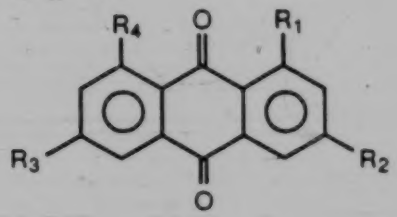
**10** $CH_3$-$(CH_2)_{14}$-COOH

**8** OH

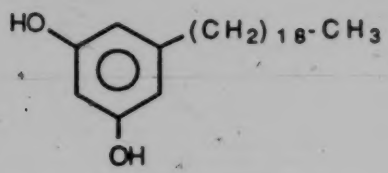
**9** OCO-$C_{15}H_{31}$



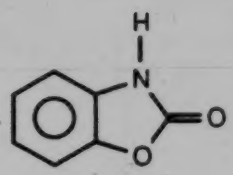
| | $R_1$ | $R_2$ |
|---|---|---|
| **11** | H | OH |
| **16** | OH | OH |
| **42** | $OCH_3$ | OH |



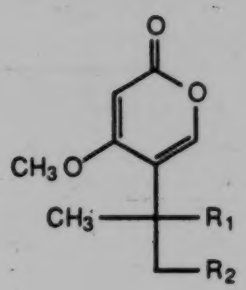
| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| **3** | OH | OH | OH | OH |
| **24** | OH | H | H | $OCH_3$ |
| **41** | OH | H | H | OH |

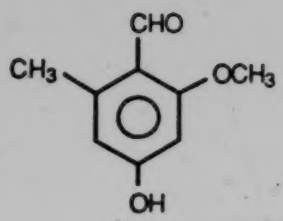


**28**

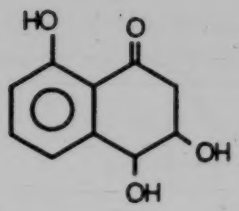


**29**





**30**



**31**

| | $R_1$ | $R_2$ |
|---|---|---|
| **35** | H | OH |
| **39** | OH | OH |

## 5. Biological Studies of Crude Extracts and Metabolites from *Verticicladiella* sp. C50 and C728

### 5.1 Test of inhibition of water conduction

It is suggested that the vascular wilt of the black stain root disease infected trees is because fungal metabolites may disrupt stem water transport[95,96]. The inhibition of water transport is assumed to relate to the phytotoxicity of fungal metabolites, although no evidence demonstrating reduced water conduction has been reported. A water uptake experiment using lodgepole pine seedlings has been developed in our laboratories[97]. The test of inhibition of water conduction is based on the assumption that the degree of water uptake in the seedlings will reflect the degree of inhibition caused by the compound being tested. One month old pine seedlings are used for the test. The seedlings are allowed to stand in the test solution for 12 hours, then they are transferred to an aqueous dye solution and the uptake of dye is observed. The results of the tests are presented in Table 27. From the data it appears that the inhibition of water conduction of the crude extracts of *Verticicladiella* sp. C50 and C728 may be due to the presence of xanthone and anthraquinone compounds. Metabolites **11**, **16**, **41**, and **42** are the most active, and compounds **3** and **24** show moderate activity. Further testing of these compounds will be carried out by scientists at the Northern Forest Research Center.

Table 27. Inhibition of water conduction test

| Sample | IDC* | | |
|---|---|---|---|
| Crude extract** | 1 | 2 | 3*** |
| C50 (rye) | II | II | II |
| C728 (rye) | II | II | II |
| C50 (Mycelium) | I | I | I |
| C50 (Broth) | Δ | Δ | Δ |
| Pure compound | 1 | 2 | 3 |
| 3 | Δ | Δ | 0 |
| 6 | 0 | 0 | 0 |
| 11 | Δ | Δ | Δ |
| 16 | Δ | Δ | I |
| 24 | Δ | Δ | 0 |
| 28 | 0 | 0 | 0 |
| 29 | 0 | 0 | 0 |
| 41 | Δ | Δ | Δ |
| 42 | Δ | Δ | Δ |

* IDC: Inhibition of dye conduction, record result as

0 no inhibition, good conduction

I slight inhibition, medium conduction

II fair inhibition, slight conduction

III complete inhibition, no conduction

Δ no conduction in young leaves.

** Total crude extracts.

*** Each sample was tested three times.

Dye: 0.1% Acid fuchsin dye.

Sample: 0.1% Aqueous solution.

## 5.2 Antibiotic screening of the metabolites from *Verticicladiella* sp. C50 and C728

The sodium salts of the anthraquinones and xanthones isolated from C50 and C728 have been tested for antibiotic activity using the agar diffusion method[98,99]. 1,3,6,8-Tetrahydroxyanthraquinone (3) and 1-hydroxy-8-methoxyanthraquinone (24) show weak antibiotic activity, since inhibition of growth of *Staphylococcus epidermidis* is observed at a concentration of 5% (Table 28). The xanthone compounds do not show activity.

Table 28. Antibiotic screening* of metabolites from *Verticicladiella* sp. C50 and C728

| Micro-organism | Pure compound | | | | |
|---|---|---|---|---|---|
| | 3 | 24 | 41 | 11 | 16 |
| *Escherichia coli* ** | 0 | 0 | 0 | 0 | 0 |
| *Staphylococcus aureus* ** | 0 | 0 | 0 | 0 | 0 |
| *Staphylococcus epidermidis* ** | 15 | 20 | 0 | 0 | 0 |
| *Candida albicans* *** | 0 | 0 | 0 | 0 | 0 |

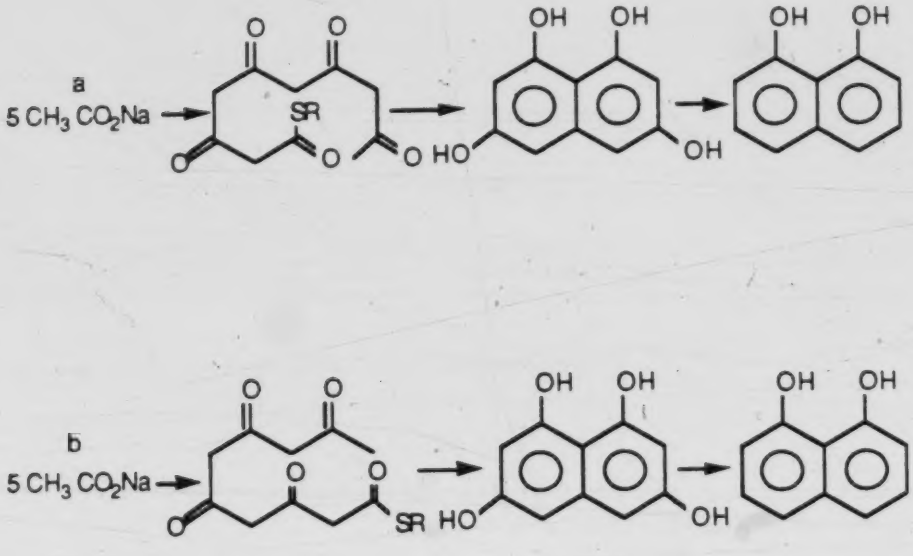
* Zone diameters of inhibition expressed in mm.

** Test organism was obtained from American Type Culture Collection (ATCC), ATCC No. *E. coli*: 25922; *S. aureus*: 25923; *S. epidermidis*: 12228.

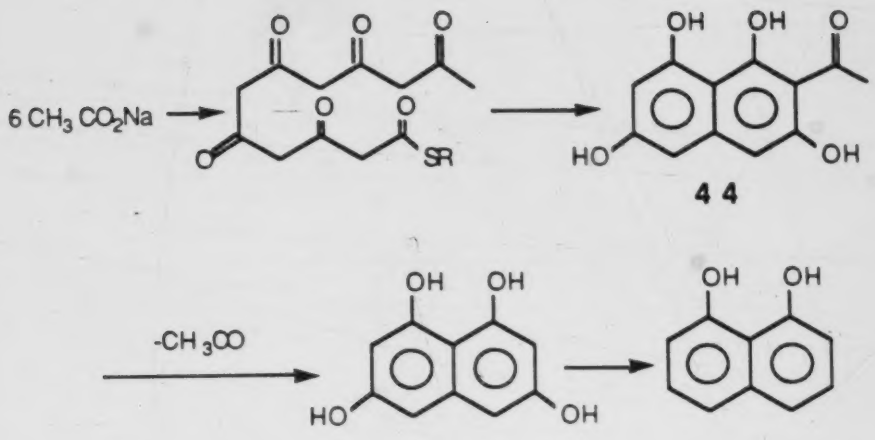
*** Test organism was obtain from University of Alberta Mold Herbarium (UAMH), UAMH No. *C. albicans*: 3468.

## 6. Biosynthetic Studies of Metabolites Produced by *Verticicladiella*

The biogenetic origin of the metabolites produced by *Verticicladiella* is an interesting topic. It is well established that naphthalenes, anthraquinones and xanthones are derived biosynthetically by a polyketide pathway. Naphthalene derivatives have been shown to be derived from either pentaketide (Scheme 13)[100-106] or hexaketide (Scheme 14)[107,108] origin, the latter biosynthetic pathway involving deacetylation of a hexaketide derivative, naphthol **44**.

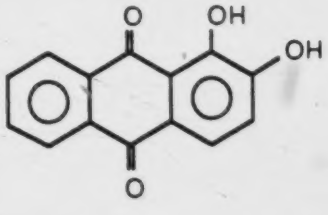



Scheme 13. Pentaketide pathway for naphthalene compounds




Scheme 14. Hexaketide pathway for naphthalene compounds

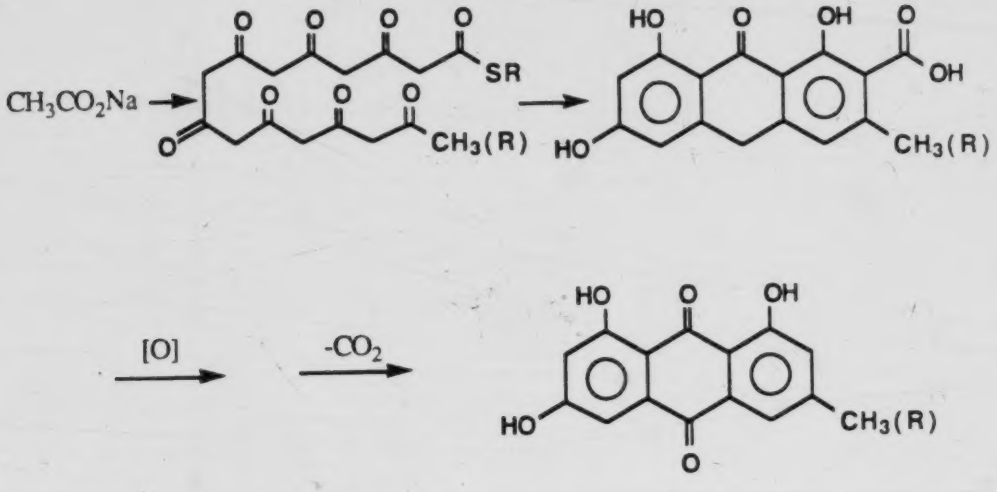
The biosynthesis of anthraquinones has been widely studied. Early investigations show that in higher plants, anthraquinones with hydroxyl groups in both rings as a general rule are derived *via* polyketides, whereas those anthraquinones with hydroxyl groups in one ring, such as alizarin (**45**), come from the shikimate and mevalonate pathway[109,110].




**45**

The biosynthesis of anthraquinones in lower organisms, however is not so clear-cut. "Nature often seems capricious in her choice of pathways"[111,112]. Many

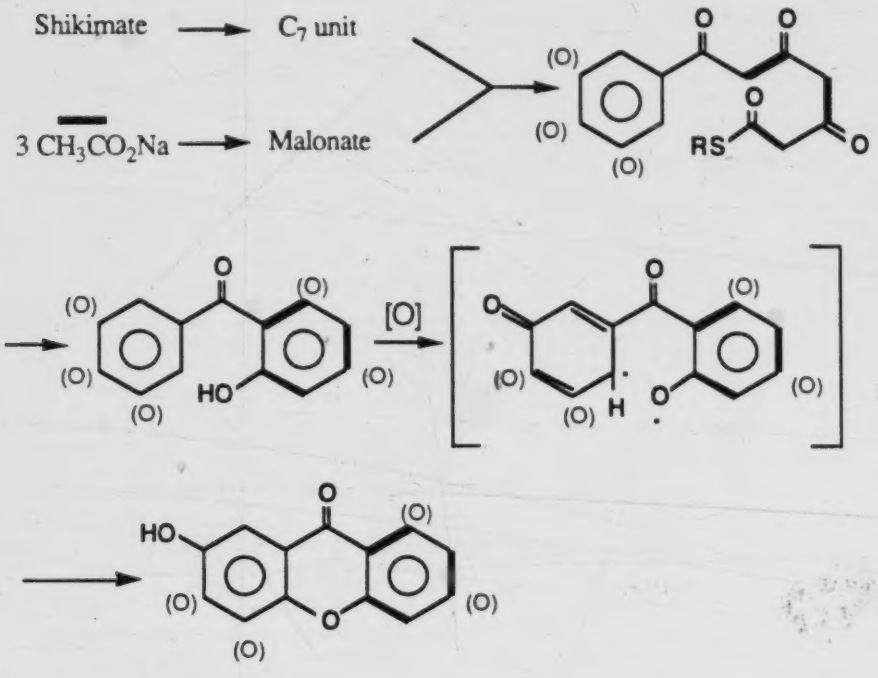
anthraquinones are formed from a polyketide pathway and most of them have at least one carbon atom attached to the ring[113-119]. The accepted polyketide biosynthetic pathway for anthraquinones is shown in Scheme 15. The anthraquinone metabolites of *Verticicladiella* do not have a carbon substitutent and this suggests that these anthraquinones may be biosynthesized by an unusual pathway.



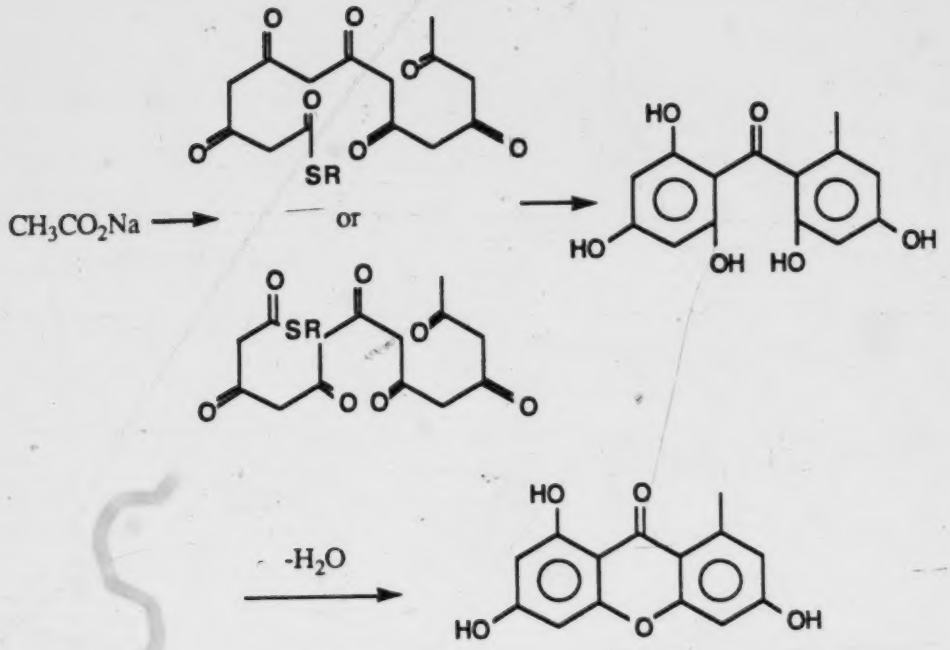

Scheme 15. Polyketide biosynthetic pathway for anthraquinones

The biosynthesis of xanthones has been studied only recently. Hydroxylated benzophenones are generally accepted as the immediate precursors of most xanthones, but their biosynthesis in higher plants and fungi usually differs[120,121]. In plants, benzophenones are derived from shikimate and acetate pathways (Scheme 16)[122-126], whereas in fungi the xanthones are almost derived solely from acetate. A "looped" folding (Scheme 17) of the fungal polyketide chain in one of two ways leads directly

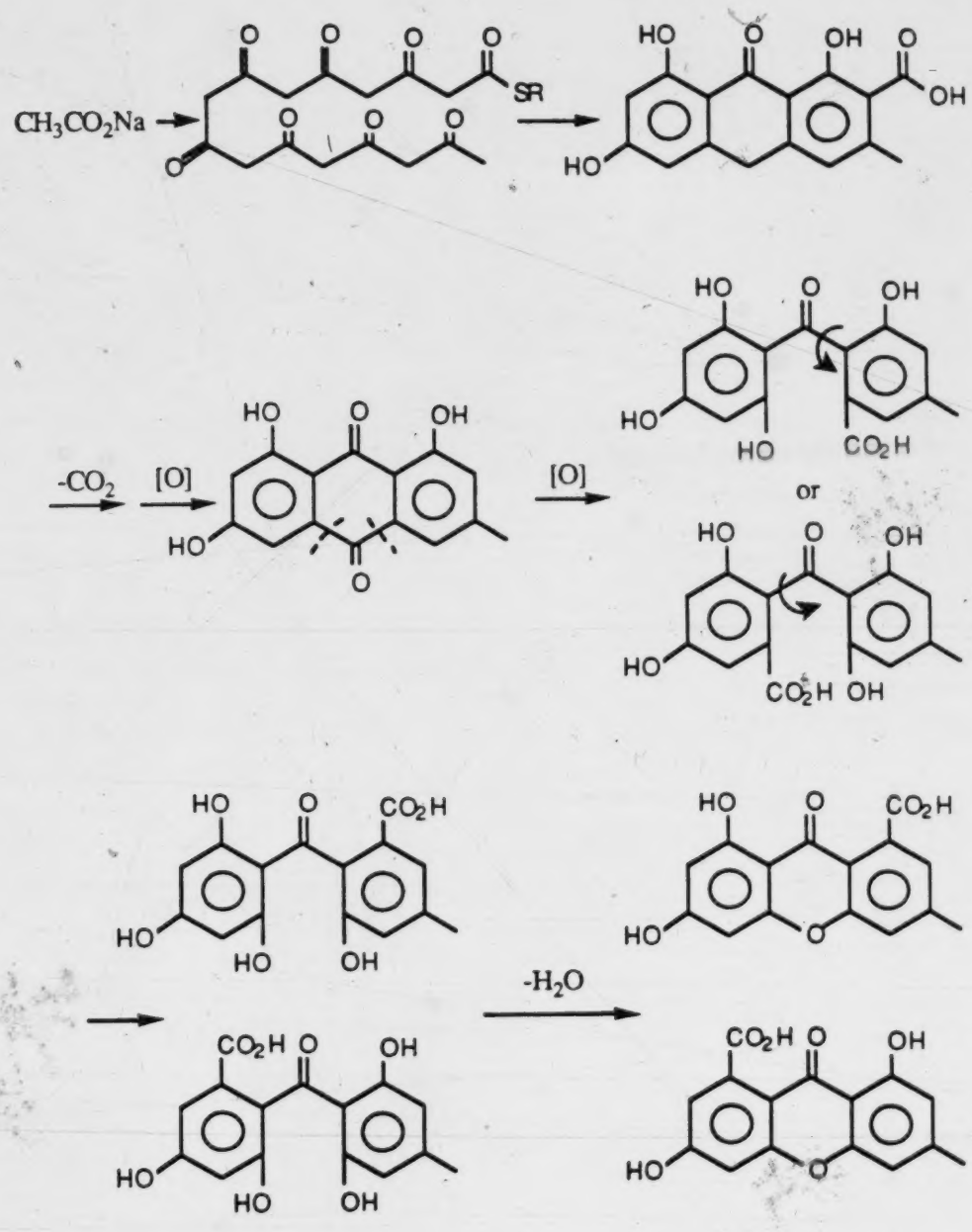
to precursors of some xanthone metabolites[127-134]. The "circular" folding (Scheme 18) requires oxidative cleavage of an intermediate anthraquinone or anthrone, and this oxidative ring fission is a common process in fungi, especially if the fungus produces a series of anthraquinone and xanthone pigments[135-142].




Scheme 16. Shikimate-acetate biosynthetic pathway for xanthones




Scheme 17. Polyketide biosynthetic pathway for xanthones

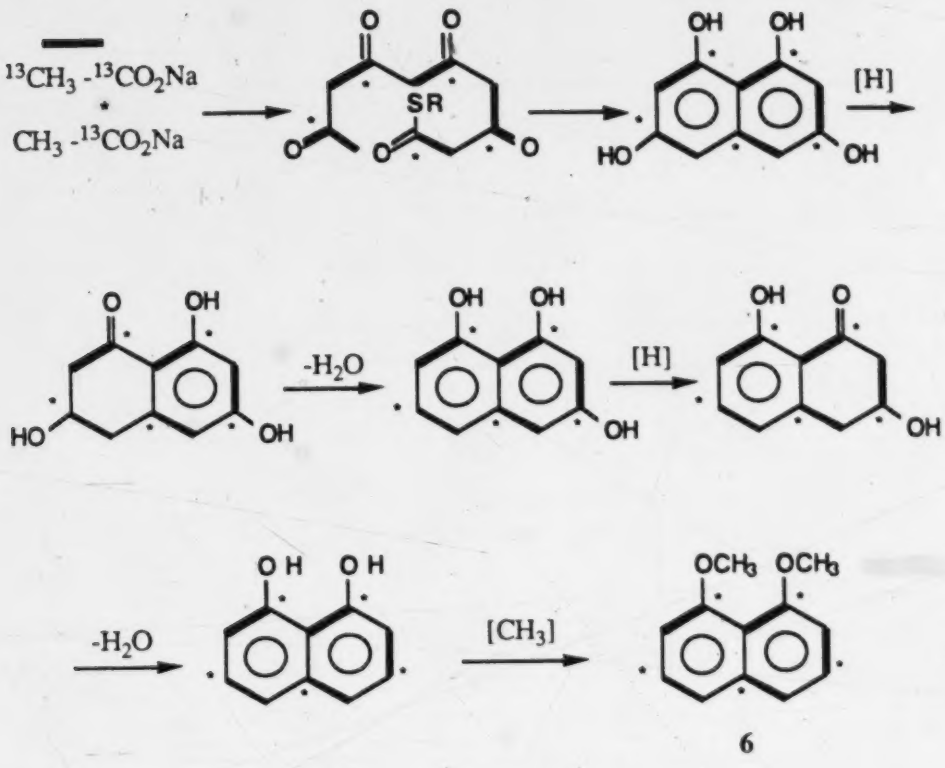



Scheme 18. Polyketide biosynthetic pathway *via* oxidative ring fission for xanthones

In order to establish the biosynthetic pathway of the metabolites produced by *Verticicladiella* , we undertook a biosynthetic study in which $^{13}C$ labelled sodium acetate was added to the culture medium. Both sodium [1-$^{13}C$] acetate and sodium [1,2-$^{13}C$] acetate were used. The singly labelled culture was grown in liquid medium (V-8 juice), whereas the doubly labelled culture was grown on solid medium (rye). The results of our studies are presented below.

6.1 Biosynthetic studies of naphthalene metabolites

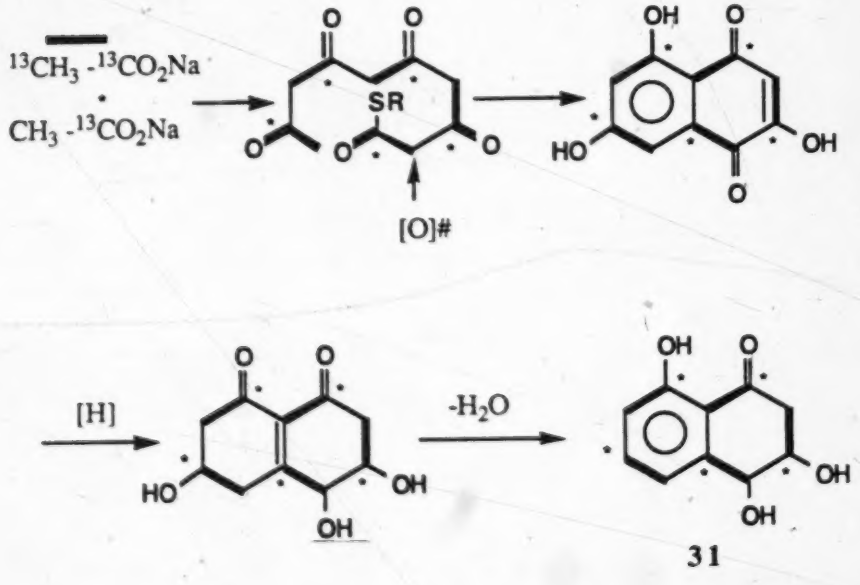
Examination of the hydrogen decoupled $^{13}C$ nmr spectrum of 1,8-dimethoxynaphthalene (6), isolated from the culture containing [1-$^{13}C$] acetate, shows enrichment (about 6% incorporation) at carbons 1, 3, 4a, 6, and 8 relative to the natural abundance spectrum. The slightly higher incorporation of $C_3$ and $C_6$ is due to "starter" effect. This suggests that the polyketide is folded so that either $C_3$-$C_4$ or $C_5$-$C_6$ is specifically derived from the starter acetate, since compound 6 has a symmetrical structure, whereas the other carbons are derived from malonate. The hydrogen decoupled $^{13}C$ nmr spectrum of 1,8-dimethoxynaphthalene (6) derived from [1,2-$^{13}C$] acetate shows that all resonances, except those of the methoxyl carbons, are accompanied by $^{13}C$-$^{13}C$ satellites. The bonded pairs of $C_1$-$C_{8a}$, $C_2$-$C_3$, $C_4$-$C_{4a}$, $C_5$-$C_6$, and $C_7$-$C_8$ are identified by matching the $^1J_{cc}$ coupling constants. This information establishes that the naphthalene nucleus is biosynthesized entirely by condensation of five intact two-carbon units. Thus, 1,8-dimethoxynaphthalene (6) is derived biosynthetically *via* a pentaketide pathway as in Scheme 19.




Scheme 19. Pentaketide biosynthetic pathway for 1,8-dimethoxynaphthalene

3,4-Dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone (**31**) is expected to be derived biosynthetically by the same pathway as 1,8-dimethoxynaphthalene (**6**). Examination of the hydrogen decoupled $^{13}C$ nmr spectrum of 3,4-dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone (**31**), isolated from the culture containing [1-$^{13}C$] acetate, reveals a labelling pattern similar to that observed for that of 1,8-dimethoxynaphthalene (**6**), i.e. carbons 1, 3, 4a, 6, and 8 are enriched (about 6% incorporation) relative to the natural abundance spectrum. $C_6$ from the starter acetate is more enriched relative to the other carbons derived *via* malonate. The hydrogen

decoupled $^{13}C$ nmr spectrum of compound **31** isolated from the culture containing [1,2-$^{13}C$] acetate shows all carbon signals accompanied by $^{13}C$-$^{13}C$ satellites. The coupling constants of the satellites indicate the five intact two-carbon units are $C_1$-$C_{8a}$, $C_2$-$C_3$, $C_4$-$C_{4a}$, $C_5$-$C_6$, and $C_7$-$C_8$. Therefore, a pentaketide biosynthetic pathway is established for the formation of 3,4-dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone (**31**) (Scheme 20).

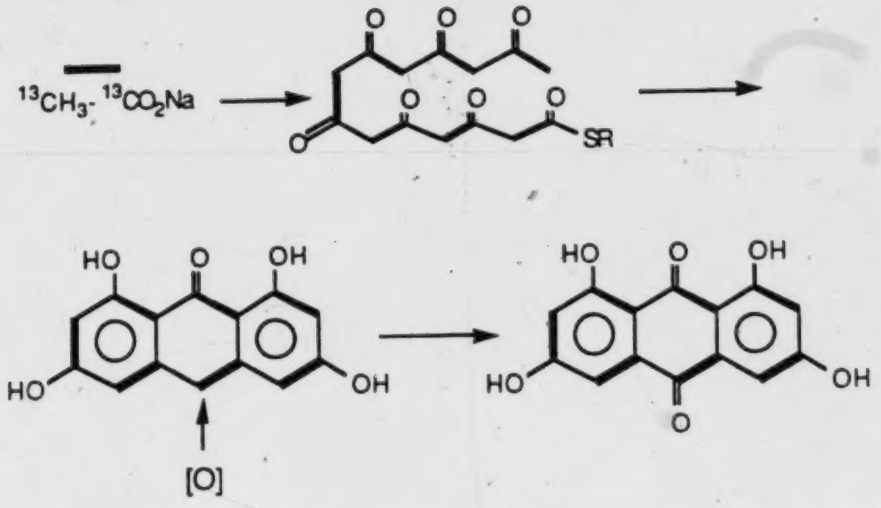



Scheme 20. Pentaketide biosynthetic pathway for 3,4-dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone

# Oxidation could take place at a later stage.

## 6.2 Biosynthetic studies for anthraquinone metabolites

It is interesting to note that anthraquinone metabolites produced by *Verticicladiella* sp. do not have a carbon atom substituent in the ring. The formation of these anthraquinones would be expected to occur either by cyclization of a normal octaketide pathway (Scheme 15) and then loss of a carbon, or by an unusual direct heptaketide pathway. Examination of the hydrogen decoupled $^{13}C$ nmr spectrum of 1,3,6,8-tetrahydroxyanthraquinone (3) enriched from [1,2-$^{13}C$] acetate (about 0.1% incorporation) shows that all signals are accompanied by $^{13}C$-$^{13}C$ satellites. These are displayed as seven coupled pairs of carbon atoms. The $^{1}J_{cc}$ values indicate that the seven intact two-carbon units are $C_1$-$C_2$, $C_3$-$C_4$, $C_{4a}$-$C_{10}$, $C_{10a}$-$C_5$, $C_6$-$C_7$, $C_8$-$C_{8a}$, and $C_9$-$C_{9a}$. This information suggests that compound 3 is probably biosynthesized directly from a heptaketide (Scheme 21). Since no such direct polyketide biosynthesis of anthraquinones has been reported to date, further biosynthetic studies have to be undertaken to confirm the proposed pathway.



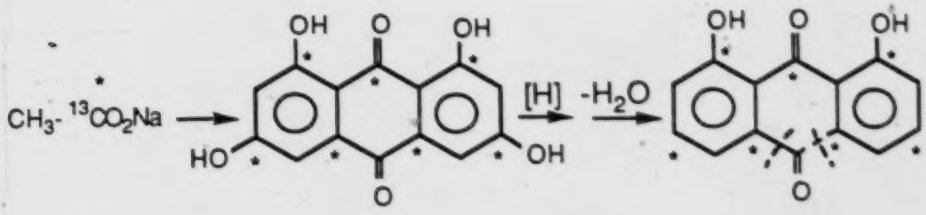

3

Scheme 21. Heptaketide biosynthetic pathway for 1,3,6,8-tetrahydroxy-anthraquinone

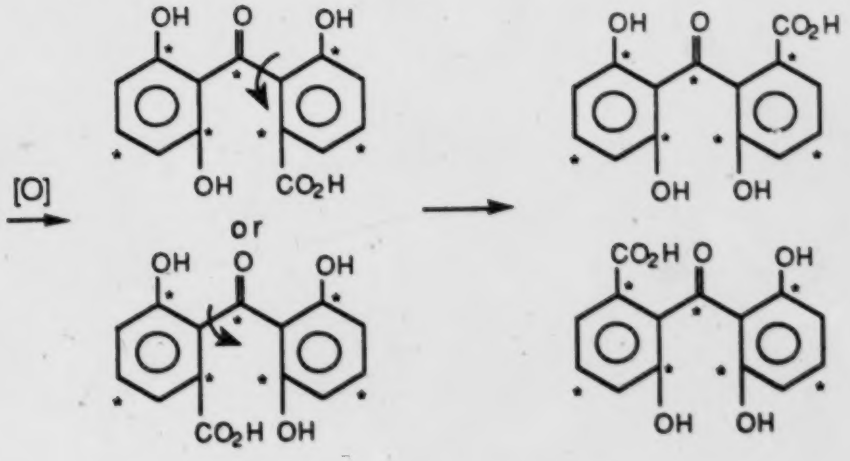
6.3 Biosynthetic studies of xanthone metabolites

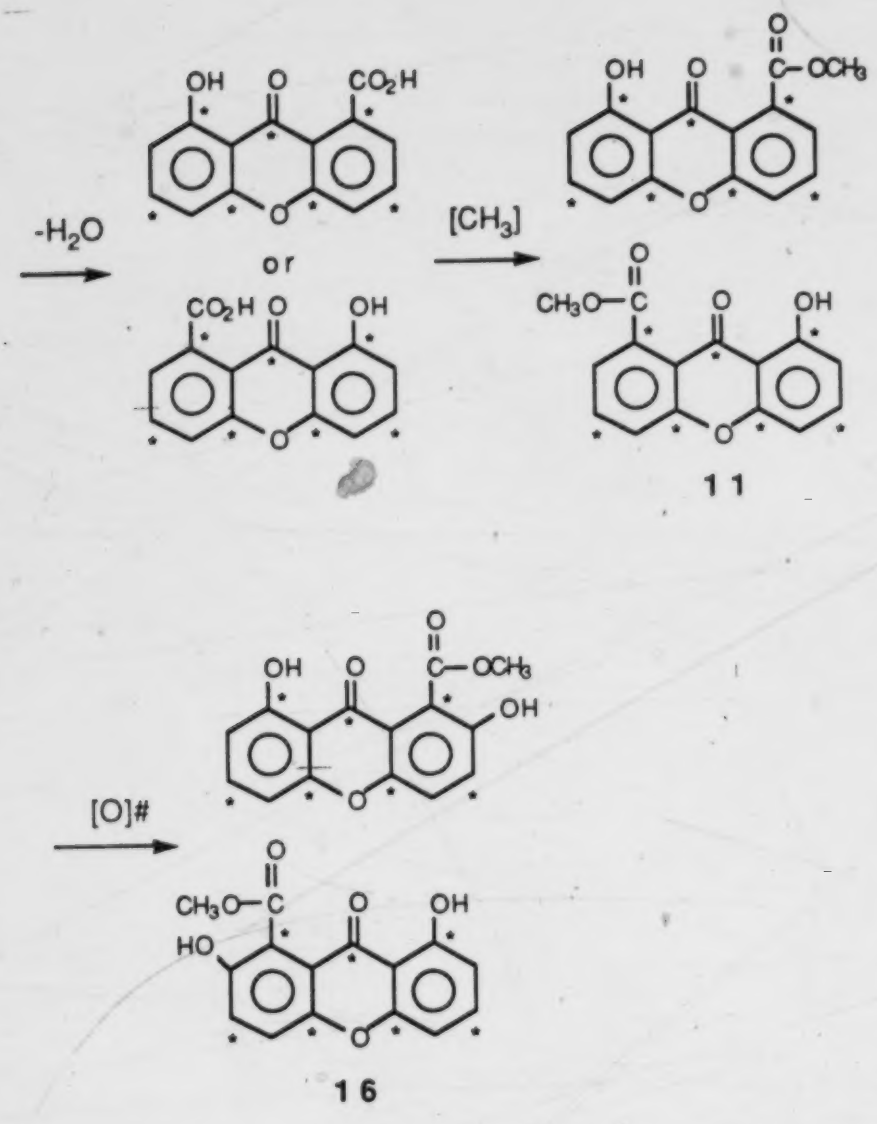
The xanthone metabolites produced by *Verticicladiella* sp. are presumably derived from oxidative fission *via* the anthraquinones isolated from *Verticicladiella* sp. In order to distinguish between the possible biosynthetic routes, both singly labelled sodium [1-$^{13}$C] acetate and doubly labelled sodium [1,2-$^{13}$C] actate were incorporated into the xanthone metabolites with growing cultures of *Verticicladiella* sp. C50. Unfortunately, the xanthone metabolites grown in the doubly labelled fashion were isolated in very small amounts and the $^{13}$C nmr experiment did not

provide additional information. Examination of the hydrogen decoupled $^{13}C$ nmr spectrum of singly labelled vertixanthone (11) shows enrichment (about 7% incorporation) at carbons 1, 3, 4a, 6, 8, 9, and 10a relative to the natural abundance spectrum. The hydrogen decoupled $^{13}C$ nmr spectrum of singly labelled hydroxyvertixanthone (16) reveals the same labelling pattern as that of vertixanthone (11). Thus, the xanthone metabolites 11 and 16 produced by *Verticicladiella* sp. are derived from polyketides *via* oxidative ring fission of anthraquinones 3 and 41 (Scheme 22). Since the intermideate benzophenones possesses an axis of symmetry in one conformationally labile ring which affords a mixture of two labelling patterns in the xanthones[143,144], the direction of cyclization of benzophenones to xanthones is unknown in this case.




3 41






Scheme 22. Biosynthetic pathway for vertixanthone and hydroxyvertixanthone

# Oxidation may occur at a early stage.

## III. EXPERIMENTAL

All solvents except diethyl ether were distilled prior to use. ACS quality anhydrous diethyl ether was used without purification. Petroleum ether refers to Skelly Oil Company light petroleum (Skellysolve B), bp 62-70°C. Pyridine was distilled from $CaH_2$ and stored over molecular sieves, acetic anhydride was dried over $P_2O_5$ and distilled from sodium acetate.

Analytical thin layer chromatography (tlc) was carried out on aluminum sheets (75x25 or 75x50) pre-coated (0.2 mm) with silica gel $60F_{254}$ (E. Merck, Darmstadt). Marterials were detected by visualization under an ultraviolet (uv) lamp (254 or 350 nm), or by spraying with a solution of phosphomolybdic acid (5%) containing a trace of ceric sulfate in aqueous sulfuric acid (5%, v/v), or a solution of anisaldehyde (2%) in aqueous sulfuric acid (10%, v/v), followed by charring on a hot plate. Flash column chromatography[145] was performed with Merck Silica Gel 60 (40-63 μm).

High resolution mass spectra (hrms) were recorded on an A. E. I. MS-50 mass spectrometer coupled to a DS 50 computer. Chemical ionization mass spectra (cims) were recorded on an A. E. I. MS-9 mass spectrometer. Ammonia was used as reagent gas. Data are reported as m/z (relative intensity). Unless diagnostically significant, peaks with intensities less than 10% of the base peak are omitted. Ultraviolet (uv) spectra were obtained on an Unicam SP 1700 ultraviolet spectrophotometer. Infared (ir) spectra were recorded on a Nicolet 7199 FT interferometer. Optical rotations were measured on a Perkin Elmer Model 141 polarimeter. $^1H$ nuclear magnetic resonance ($^1H$ nmr) spectra were measured on a Bruker WH-360 spectrometer or a Bruker WH-400 spectrometer. $^{13}C$ nuclear magnetic resonance ($^{13}C$ nmr) spectra were measured on a Bruker WH-300 spectrometer or a Bruker WH-400 spectrometer. For $^1H$ nmr, residue $C\underline{H}Cl_3$ in $CDCl_3$ or $C\underline{H}_3OH$ in $CD_3OD$ was employed as the internal standard (assigned as 7.27 ppm or 3.30 ppm downfield from tetramethysilane (TMS)) and measurements are reported in ppm downfield from TMS (δ). For $^{13}C$ nmr, $CDCl_3$ or

$CD_3OD$ was employed as the internal standard (assigned as 77.00 ppm or 49.00 ppm downfield from TMS) and measurements are reported in ppm downfield from TMS (δ). Melting points were recorded on a Fisher-Johns melting point apparatus and are uncorrected. The strain of *Verticicladiella* species C728 (isolated from Douglas fir, Radium Hot Springs, B. C.) used in this study was obtained from Y. Hiratsuka, Northern Forest Research Center (NFRC), Edmonton. The strain of *Verticicladiella* species C50 (isolated from lodgepole pine) used in the study was supplied by C. L. K. Paulsen, Pacific Forest Research Center (PFRC), Victoria.

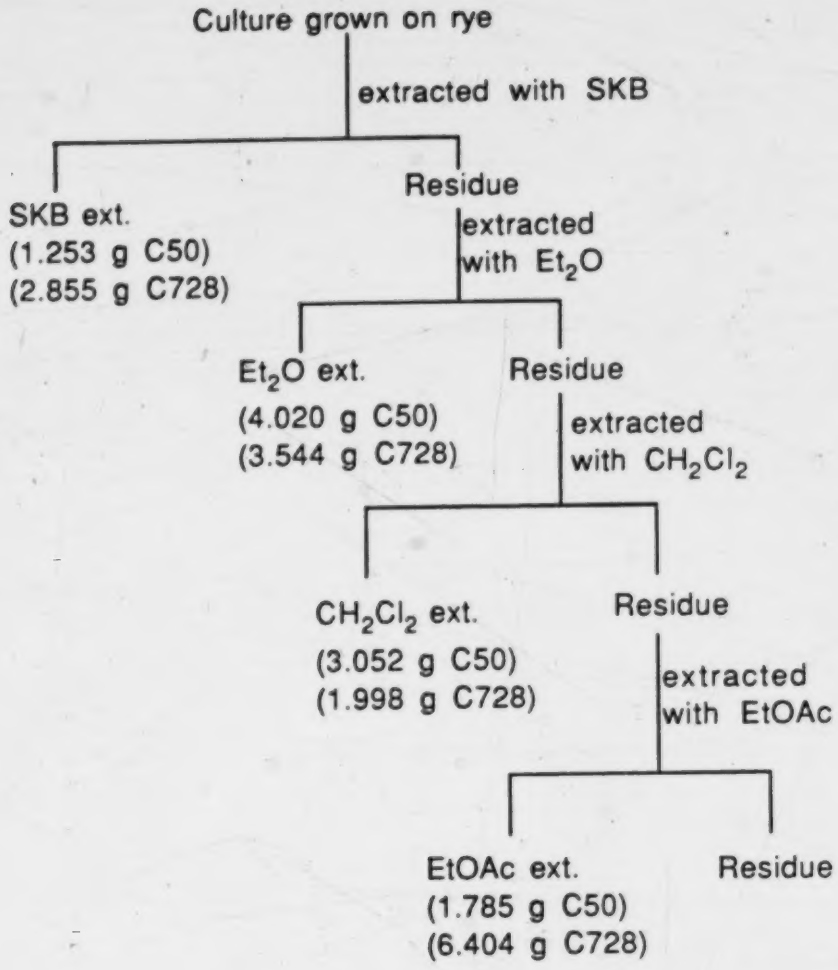
1. Growth of *Verticicladiella* sp. C50 and C728 on Solid Media and Extraction of the Metabolites

1.1 Growth of *Verticicladiella* sp. C50 and C728 on solid media

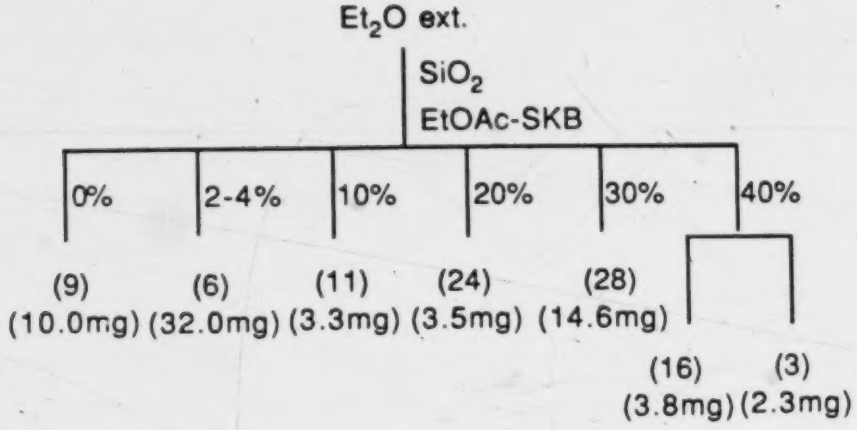
Winter rye (500g) was soaked in warm tap water (400g) for 8-12 hours. Excess water was drained off, the solid substrate was placed in five autoclavable plastic bags, and the media were autoclaved twice for 30 minutes at 121°C. An aqueous suspension of mycelium of *Verticicladiella* sp. C50 or C728 was used to inoculate an agar plate (10% filtered V-8 juice, 1% glucose, 2% agar). After 7-10 days at room temperature, the plate culture was blended with sterile water (250 mL). The mycelial suspension (25 mL) was inoculated to each bag of solid media, and the culture was kept at room temperature for six weeks.

1.2 Extraction of the metabolites

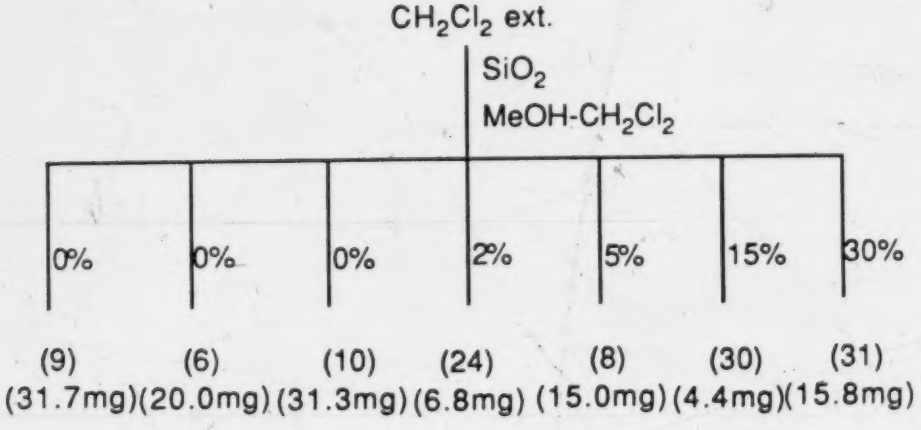
The culture of *Verticicladiella* sp. C50 or C728 grown on solid media was extracted successively in a Soxhlet extractor with Skellysolve B, ether, methylene chloride, and ethyl acetate. Each solvent extraction was carried out for 24 hours, then the extract was concentrated on rotary evaporator (Scheme 23). Each of the four solvent crude extracts was separated by flash chromatography utilizing gradient elution with either an ethyl acetate-Skellysolve B or a methanol-methylene chloride solvent system. The metabolites isolated from crude extracts are listed in Scheme 24-30. The crude ether extract of C50 was also separated into acidic, basic and neutral fractions in the manner described in Scheme 31. The uninoculated rye medium (blank) was subjected to a similar extraction procedure. Only fatty acids and triglycerides were isolated. The fatty acids and triglycerides obtained from the crude extracts are identical with those produced by the rye blank. The fractions from chromatography were further purified as described for the characterization of individual compounds.



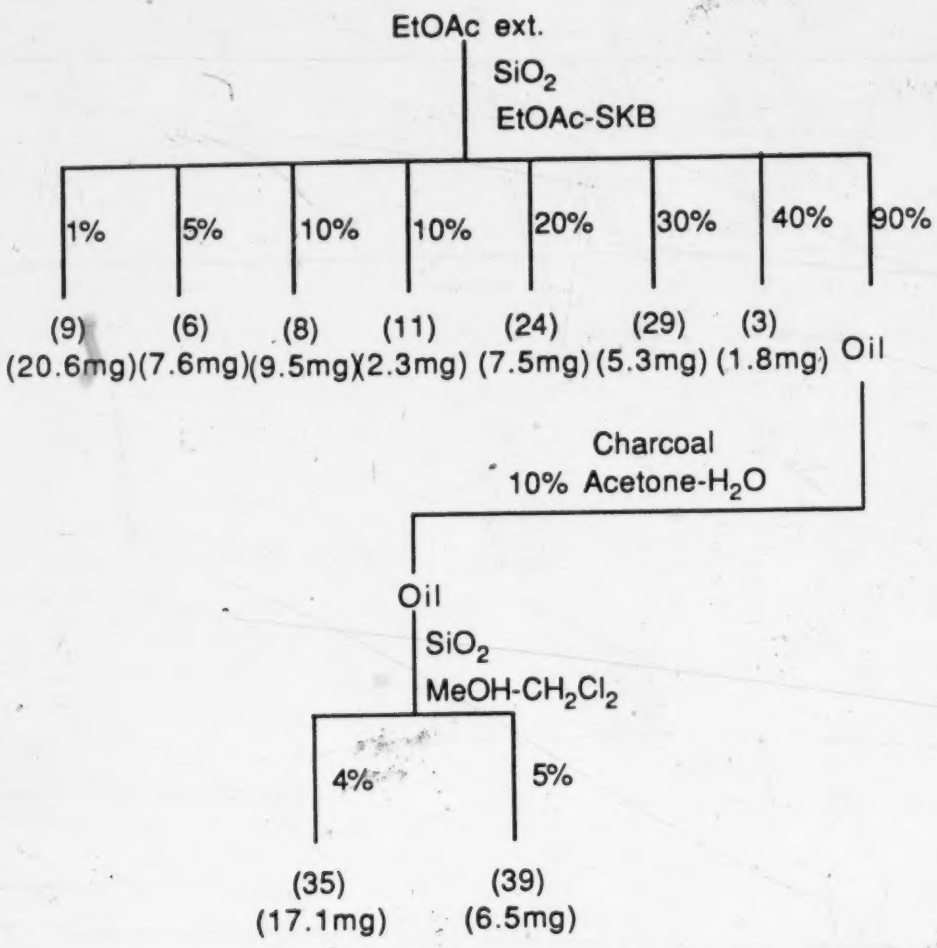

Scheme 23. Extraction of solid media cultures of *Verticicladiella*



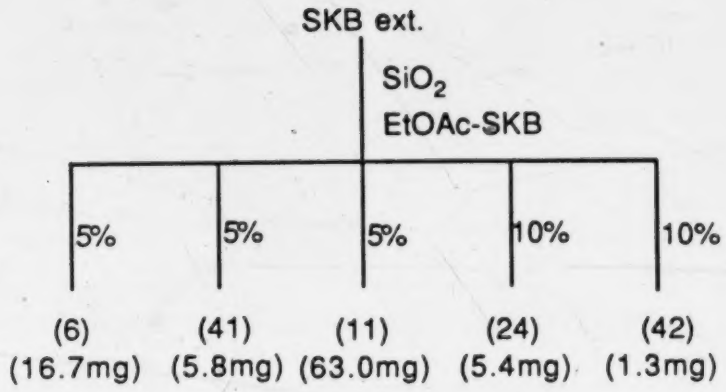

Scheme 24. Metabolites of C50 (ether extract)



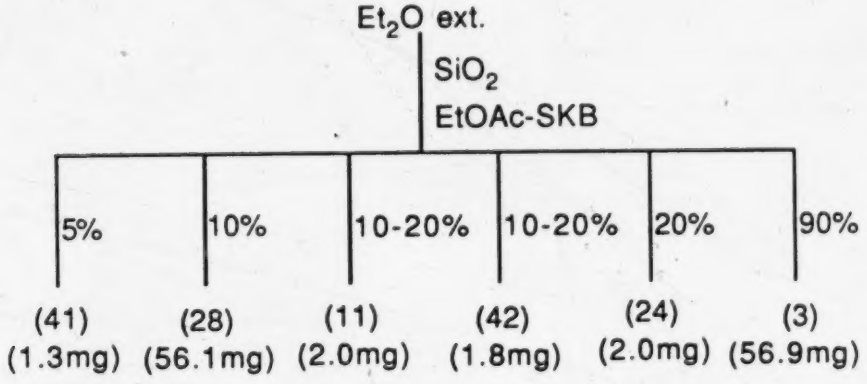

Scheme 25. Metabolites of C50 (methylene chloride extract)



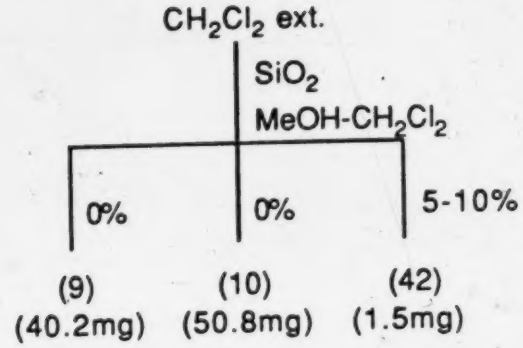

Scheme 26. Metabolites of C50 (ethyl acetate extract)



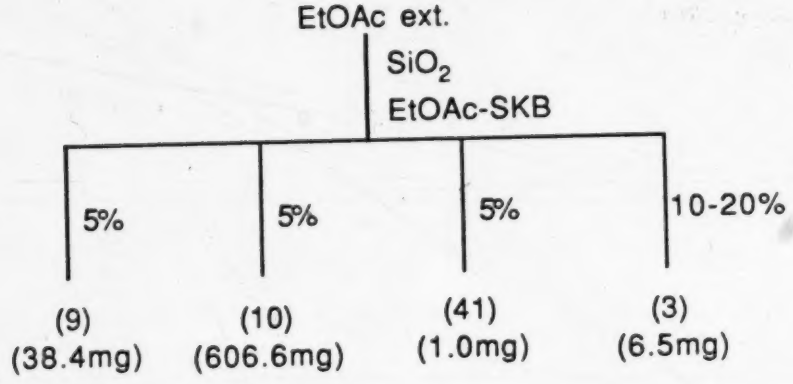

Scheme 27. Metabolites of C728 (Skellysolve B extract)



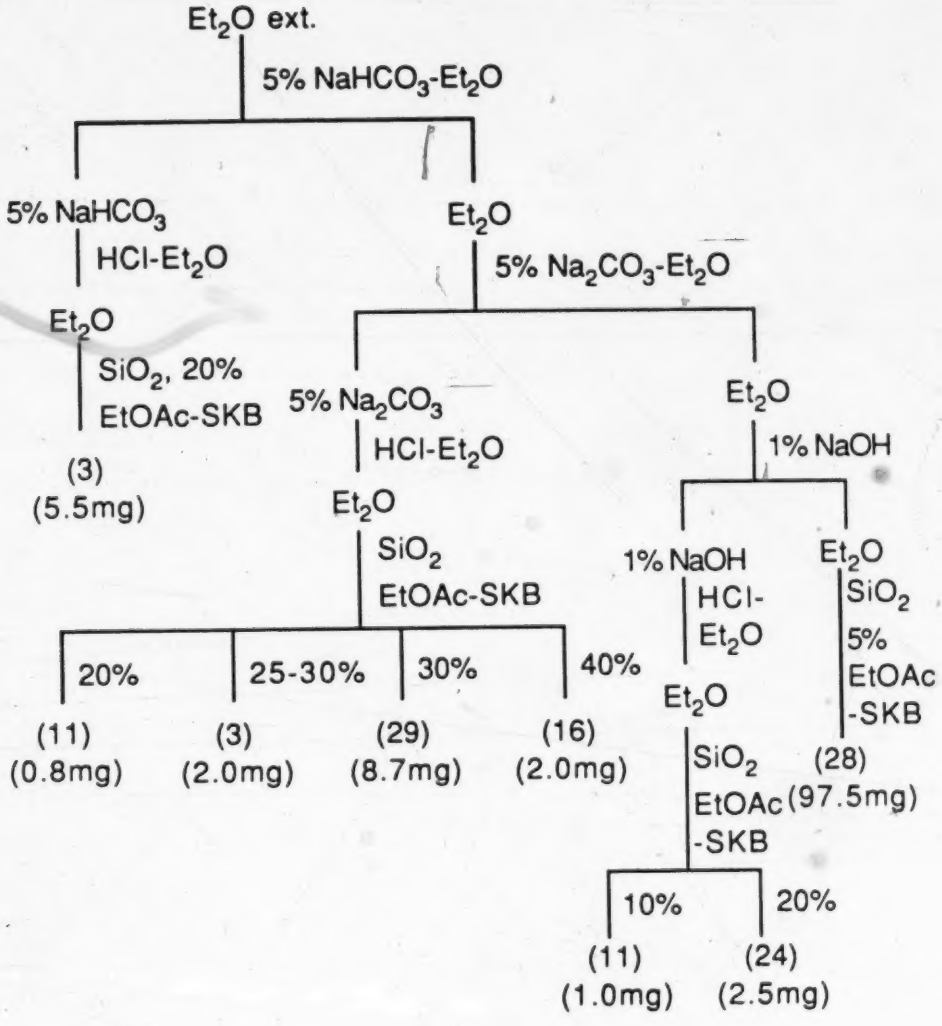

Scheme 28. Metabolites of C728 (ether extract)




Scheme 29. Metabolites of C728 (methylene chloride extract)




Scheme 30. Metabolites of C728 (ethyl acetate extract)

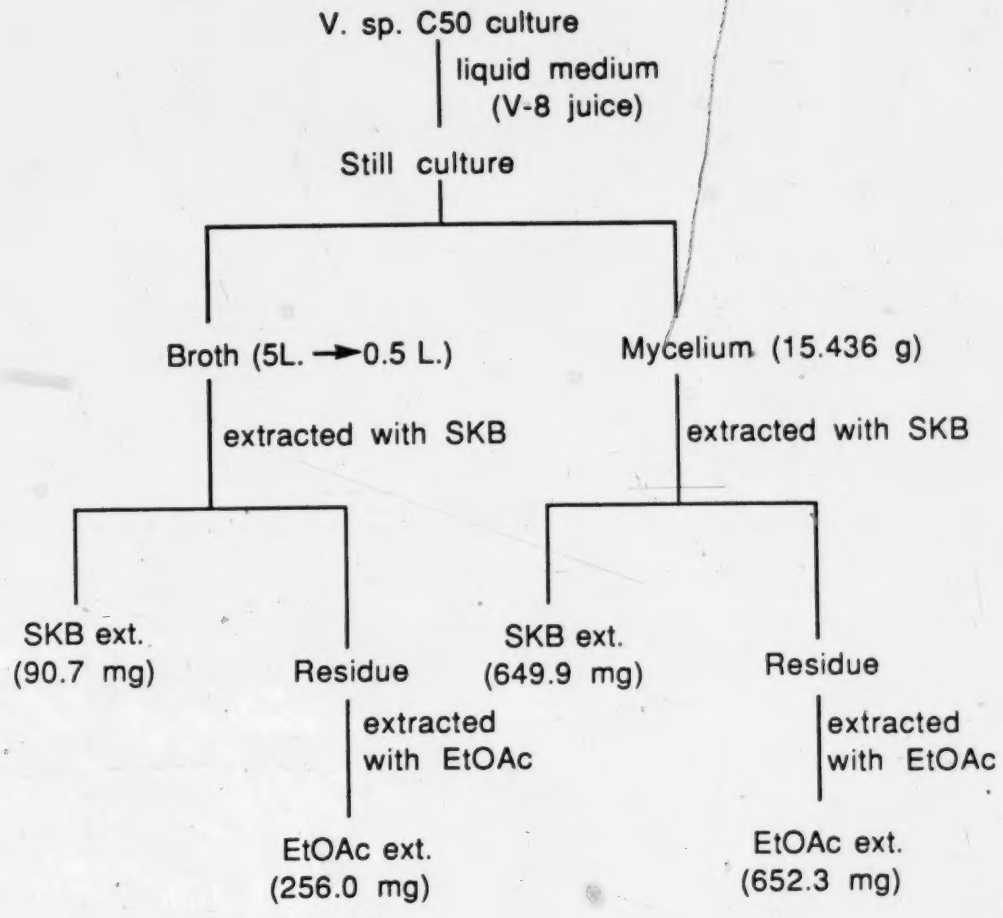



Scheme 31. Separation of ether extract of strain C50 into acidic, basic and neutral fractions

## 2. Growth of *Verticicladiella* sp. C50 in Liquid Medium and Extraction of the Metabolites

### 2.1 Growth of *Verticicladiella* sp. C50 in liquid medium

Cultures of *Verticicladiella* sp. C50 were maintained at 4°C in slant tubes containing Difco potato dextrose agar. An aqueous suspension of mycelium was used to inoculate two agar plates (10% filtered V-8 juice, 1% glucose, 2% agar). After 7-10 days at room temperature, the culture was blended in a Waring blender with *ca.* 200 mL sterile media (10% filtered V-8 juice, 1% glucose) and *ca.* 20 mL aliquots were used to inoculate 5x1 L sterile medium in 2 L flasks. After inoculation the still cultures were kept at room temperature for six weeks. The culture broth was decanted from the mycelium, concentrated *in vacuo* to *ca.* 500 mL and continuously extracted with Skellysolve B then ethyl acetate for 24 hours. The organic extracts were dried and concentrated to give an oil which was separated as described below. The mycelium was subjected to successive continuous extraction in a Soxhlet extractor with Skellysolve B and ethyl acetate (Scheme 32). The organic extracts were dried, concentrated and separated as described below.



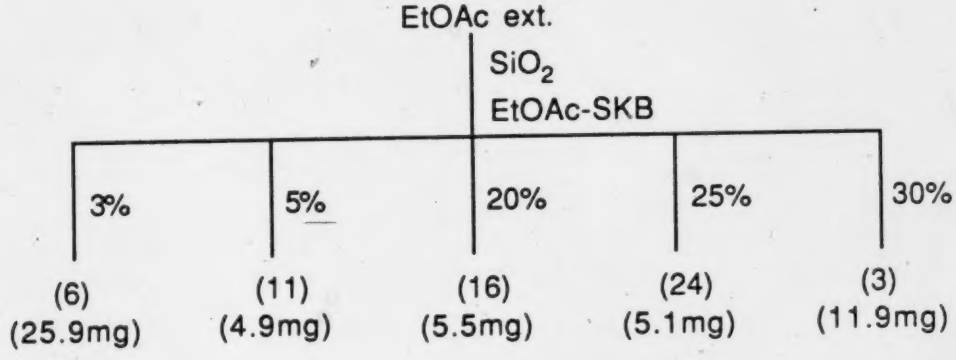

Scheme 32. Extraction of liquid still culture of *Verticicladiella* sp. C50

2.2 Extraction of the metabolites

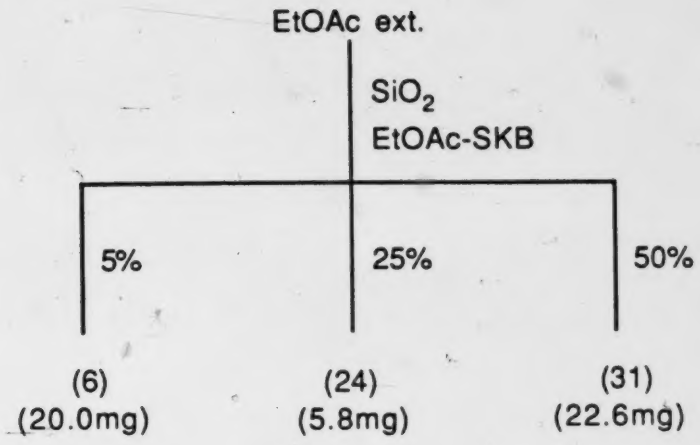
The crude mycelium extracts were separated by flash chromatography over silica gel using gradient elution with ethyl acetate in Skellysolve B. 1,8-Dimethoxynaphthalene (**6**) (425.5 mg) was obtained in the 3% ethyl acetate in

Skellysolve B fraction of the crude Skellysolve B extract (649.9 mg). From the crude ethyl acetate extract (652.3 mg), 1,8-dihydroxyanthraquinone (**3**) 1,8-dimethoxy-naphthalene (**6**), vertixanthone (**11**), hydroxyvertixanthone (**16**), and 1-hydroxy-8-methoxyanthraquinone (**24**) were isolated as shown in Scheme 33.

Separation of the crude broth Skellysolve B extract (90.7 mg) by silica gel flash chromatography (eluent: 5% ethyl acetate in Skellysolve B) gave 1,8-dimethoxynaphthalene (**6**) (38.2 mg). The crude broth ethyl acetate extract (256.0 mg) was subjected to gradient flash chromatography over silica gel with ethyl acetate in Skellysolve B. Three metabolites, 1,8-dimethoxynaphthalene (**6**), 1-hydroxy-8-methoxyanthraquinone (**24**), and 3,4-dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone (**31**), were isolated as shown in Scheme 34.




Scheme 33. Metabolites isolated by chromatography of the mycelium ethyl acetate extract




Scheme 34. Metabolites isolated by chromatography of the broth ethyl acetate extract

3. Metabolites from *Verticicladiella* sp. C50 and C728 Grown on Solid Media

3.1 1,8-Dimethoxynaphthalene (6)

1,8-Dimethoxynaphthalene, was isolated from the chromatographic fractions eluted with 2-4 % ethyl acetate in Skellysolve B. It crystallized from Skellysolve B after evaporation of the solvents . Recrystallization from 95% ethanol gave 1,8-dimethoxynaphthalene (6) as white crystals (59.6 mg from C50 and 16.7 mg from C728), mp 154-155°C (157-158°C, lit[23]); tlc: $R_f$ 0.63 (Skellysolve B-ethyl acetate 3 : 2); uv (95% EtOH, 1.0 mg/100 mL) $\lambda_{max}$ nm (log $\varepsilon$): 298 (3.96), 310 (3.81), 315 (3.90), 330 (3.92); ir ($CHCl_3$, cast) $\upsilon_{max}$ $cm^{-1}$: 1580, 1480, 1432, 1375, 1238, 1091, 1054; $^1H$ nmr ($CDCl_3$, 400 MHz): $\delta$ 7.41 (2H, dd, J = 1.4, 7.1 Hz, H-4, H-5), 7.38 (2H, t, J = 7.1 Hz, H-3, H-6), 6.86 (2H, dd, J = 1.4, 7.1 Hz, H-2, H-7), 3.98 (6H, s, 2x$OC\underline{H}_3$); $^{13}C$ nmr ($CDCl_3$, 75 MHz): $\delta$ 157.12 (2C, s, C-1, C-8), 137.41 (1C, s,

C-4a), 126.37 (2C, d, C-3, C-6), 120.86 (2C, d, C-4, C-5), 117.23 (1C, s, C-8a), 106.23 (2C, d, C-2, C-7), 56.46 (2C, q, 2x$O\underline{C}H_3$); hrms m/z (relative intensity % ) calc. for $C_{12}H_{12}O_2$ ($M^+$): 188.0838; found: 188.0843 (100), 173 (5), 145 (11), 115 (47).

Methylation of 1,8-dihydroxynaphthalene:

Dimethyl sulfate was added in small portions to a stirred solution of 1,8-dihydroxynaphthalene (50 mg) in freshly prepared 10% KOH / EtOH. The mixture was refluxed for 10 minutes. Further potassium hydroxide was added, then dimethyl sulfate was added until the solution was acidic. The mixture was refluxed for 10 minutes. This procedure was repeated twice. The products were isolated by extraction with benzene. The benzene extract was washed with water, dried with magnesium sulfate, the solvents were removed, and the residue was purified by chromatography (short silica gel column (pipette), eluted with benzene). The pure fractions (tlc) were collected and the solvent was evaporated. 1,8-Dimethoxynaphthalene (22.0 mg, 38% yield) was recrystallized from 95 % ethanol. The spectral data of both synthetic and natural 1,8-dimethoxynaphthalene are identical.

3.2 β-Sitosterol (8)

β-Sitosterol (8) was isolated by silica gel flash chromatography eluting with 5-10% ethyl acetate in Skellysolve B. Recrystallization from 95% ethanol gave white crystalline needles of compound 8 (34.5 mg from C50), mp 142-143°C (143°C, lit[27]); tlc: $R_f$ 0.60 (Skellysolve B-ethyl acetate 3:2); $[\alpha]_D$ -22.5° (c, 0.20, $CHCl_3$); ir ($CHCl_3$, cast) $\upsilon_{max}$ $cm^{-1}$: 3360 (br), 1454, 1358, 1060; $^1H$ nmr ($CDCl_3$, 400 MHz): δ 5.36 (1H, m, H-6), 3.54 (1H, s, -O$\underline{H}$), 3.51 (1H, m, H-3), 2.28-1.85 (6H, m, 2xH-2, 2xH-4, 2xH-7), 1.58-0.52 (41H, m); hrms m/z (relative intensity % ) calc. for

$C_{29}H_{50}O$ ($M^+$): 414.3864; found: 414.3870 (100), 399 (18), 396 (28), 381 (14), 329 (20), 303 (25), 273 (17), 255 (18), 231 (13), 213 (18), 161 (19), 159 (18).

3.3 β-Sitosteryl palmitate (9)

Impure fractions containing one major compound were obtained by flash chromatography over silica gel with Skellysolve B. The fractions were evaporated under reduced pressure. The residue was dissolved in hot acetone and recrystallized from acetone. White platelets were identified as β-sitosteryl palmitate (9) (62.3 mg from C50, and 78.6 mg from C728), mp 92-93°C (92-94°C, lit[23]); tlc: $R_f$ 0.16 ( Skellysolve B); $[\alpha]_D$ -12.8° (c, 0.25, $CHCl_3$); ir ($CHCl_3$, cast) $\upsilon_{max}$ $cm^{-1}$: 1740, 1460, 1380, 1180, 720; $^1H$ nmr ($CDCl_3$, 400 MHz): δ 5.36 (1H, m, H-6), 4.64 (1H, m, H-3), 2.31-0.52 (78H, m); $^{13}C$ nmr ($CDCl_3$, 75 MHz): δ 173.36 (s, CO), 139.76 (s, C-5), 122.61 (d, C-6), 73.70 (d, C-3), 56.74 (d), 56.10 (d), 50.09 (d), 45.90 (d), 36.20 (d), 31.97 (d), 29.22 (d), 19.85 (q), 19.36 (q), 19.08 (q), 18.82 (q), 14.15 (q), 12.03 (q), 11.90 (q), 42.36-18.82 (25C, t, and 2C, s); hrms m/z (relative intensity %) calc. for $C_{45}H_{80}O_2$ ($M^+$): 652.6162; found: 652.6167 (9), 396 (100), 381 (10), 256 (8), 255 (20), 213 (11), 159 (11), 145 (20), 133 (14), 107 (22), 95 (28), 81 (36), 69 (28), 57 (51), 35 (35).

3.4 Hydrolysis of β-sitosteryl palmitate

Pure β-sitosteryl palmitate (9) (5.0 mg) was dissolved in methylene chloride and 10% methanolic potassium hydroxide (excess) was added. The mixture was refluxed for two and one half hours. Water was added to the reaction solution and two layers were separated.

The methylene chloride extract was washed with water until the pH was about 7, then dried over anhydrous magnesium sulfate and the solvents were evaporated. The residue was dissolved in hot acetone and crystallized to give white needles of β-sitosterol (8) (2.8 mg, 88% yield).

The aqueous extract was acidified with 1N hydrochloric acid, then extracted with methylene chloride. The methylene chloride extract was dried with anhydrous magnesium sulfate. After evaporation of the solvent, a white solid (1.2 mg, 61% yield) which was identified as palmitic acid (10), was obtained.

Both products were identified by comparison of their spectral data with that of authentic samples.

3.5 Palmitic acid (10)

Palmitic acid (10), separated by flash chromatography with Skellysolve B, was precipitated as a white solid from 95% ethanol (131.3 mg from C50 and 656.6 mg from C728), mp 46-52°C; tlc: $R_f$ 0.61 (Skellysolve B-ethyl acetate 3:2); ir ($CHCl_3$, cast) $\upsilon_{max}$ $cm^{-1}$: 3200-2500 (br), 1713, 1467, 1400, 778; $^1H$ nmr ($CDCl_3$, 400 MHz): δ 2.31 (2H, t, J = 7.5 Hz, 2xH-2), 1.60 (2H, m, 2xH-3), 1.22 (24H, m), 0.85 (3H, t, J = 7.0 Hz, -$CH_3$); hrms m/z (relative intensity %) calc. for $C_{16}H_{32}O_2$ ($M^+$): 256.2402; found: 256.2406 (86), 213 (21), 185 (13), 171 (12), 157 (14), 129 (42), 115 (15), 97 (18), 87 (19), 85 (29), 73 (100), 71 (48), 69 (41), 60 (62), 57 (82), 55 (68).

3.6 Vertixanthone (11)

Fractions containing vertixanthone were separated by silica gel flash chromatography by elution with 10% ethyl acetate in Skellysolve B. A yellow solid

precipitated from the concentrated fractions. Recrystallization from EtOAc-Skellysolve B gave yellow crystals of vertixanthone (11) (7.4 mg from C50 and 65.0 mg from C728), mp 152-154°C; tlc: $R_f$ 0.47 (Skellysolve B-ethyl acetate 3:1), $R_f$ 0.37 (Skellysolve B-benzene-methanol 1:1:0.2); uv (95% EtOH, 1.0 mg/100 mL) $\lambda_{max}$ nm (log ε): 232 (4.61), 264 (4.60), 290 (4.50), 384 (3.72); ir ($CHCl_3$, cast), $\upsilon_{max}$ $cm^{-1}$: 3100 (br, OH, hydrogen-bonded), 1730 (ester), 1640 (CO, hydrogen-bonded), 1610, 1600, 1570, 1460, 1280, 1200, 1130, 810; $^1H$ nmr ($CDCl_3$, 400 MHz): δ 12.24 (1H, s, O<u>H</u>), 7.77 (1H, t, J = 8.0 Hz, H-3), 7.61 (1H, t, J = 8.0 Hz, H-6), 7.56 (1H, dd, J = 1.0, 8.0 Hz, H-2), 7.33 (1H, dd, J = 1.0, 8.0 Hz, H-4), 6.94 (1H, dd, J = 1.0, 8.0 Hz, H-5), 6.82 (1H, dd, J = 1.0, 8.0 Hz, H-7), 4.05 (3H, s, OC<u>H</u>$_3$); $^{13}C$ nmr ($CDCl_3$, 75 MHz): δ 181.07 (s, C-9), 169.60 (s, C-11), 161.86 (s, C-10a), 156.11 (s, C-4a), 155.85 (s, C-8), 137.20 (d, C-6), 135.08 (d, C-3), 133.73 (s, C-1), 122.72 (d, C-2), 119.53 (d, C-4), 117.58 (s, C-9a), 111.00 (d, C-5), 109.04 (s, C-8a), 106.94 (d, C-7), 53.18 (q, O<u>C</u>H$_3$); hrms m/z (relative intensity %) calc. for $C_{15}H_{10}O_5$ ($M^+$): 270.0528; found: 270.0533 (43), 239 (33), 238 (100), 210 (46), 182 (8), 155 (18), 126 (35), 75 (22), 63 (21), 51 (11).

### 3.7 Acetylation of vertixanthone

Acetic anhydride (0.25 mL) was added into a solution of vertixanthone (11) (4.0 mg) in pyridine (0.5 mL). The reaction mixture was stirred at room temperature overnight. Toluene (5.0 mL) was added to the solution and the solution was evaporated under reduced pressure. The residue was recrystallized from Skellysolve B. A colourless, crystalline monoacetate derivative, compound **15**, (2.5 mg, 54% yield) was obtained, mp 170°C (decomposed); tlc: $R_f$ 0.51 (Skellysolve B-ethyl acetate 1:1); ir ($CHCl_3$, cast) $\upsilon_{max}$ $cm^{-1}$: 1764 (ester), 1730 (ester), 1659 (CO), 1620, 1602, 1291, 1010; $^1H$ nmr ($CDCl_3$, 300MHz): δ 7.66 (1H, t, J = 8.5 Hz, H-3), 7.64 (1H, t,

J = 8.5 Hz, H-6), 7.48 (1H, dd, J = 1.0, 8.5 Hz, H-2), 7.35 (1H, dd, J = 1.0, 8.5 Hz, H-4), 7.25, (1H, dd, J = 1.0, 8.5 Hz, H-5), 6.95 (1H, dd, J = 1.0, 8.5 Hz, H-7), 3.93 (3H, s, $OCH_3$), 2.38 (3H, s, $OCOCH_3$); $^{13}C$ nmr ($CDCl_3$, 75 MHz): δ 174.51 (s, C-9), 169.91 (s, $OCOCH_3$), 169.69 (s, C-11), 156.88 (s, C-10a), 155.27 (s, C-4a), 150.11 (s, C-8), 134.76 (d, C-6), 134.28 (d, C-3), 134.08 (s, C-1), 123.00 (d, C-2), 119.71 (s, C-9a), 119.38 (d, C-4), 118.57 (d, C-5), 116.01 (d, C-7), 115.23 (s, C-8a), 52.59 (q, $OCH_3$), 21.21 (q, $OCOCH_3$); hrms m/z (relative intensity %) calc. for $C_{17}H_{12}O_6$ ($M^+$): 312.0634; found: 312.0639 (13), 270 (59), 239 (25), 238 (100), 210 (14), 126 (9).

3.8 Hydroxyvertixanthone (16)

The chromatographic fraction (40% ethyl acetate in Skellysolve B) from the ether crude extract consisted of two components. Fractional crystallization from acetone gave yellow crystals of hydroxyvertixanthone (16) (5.8 mg from C50), mp 244-245°C; tlc: $R_f$ 0.55 (methylene chloride-methanol 9.5:0.5). $R_f$ 0.35 (Skellysolve B-ethyl acetate 3:1); uv (95% EtOH, 1.0 mg/100 mL) $\lambda_{max}$ nm (log ε): 238 (4.14), 264 (4.25), 290 (3.71), 390 (3.50); ir ($CHCl_3$, cast) $\upsilon_{max}$ $cm^{-1}$: 3300-3100 (br, OH), 1706 (ester), 1640 (CO, hydrogen-bonded), 1605, 1582, 1440, 1380, 1290, 1222, 1050, 817, 740; $^1H$ nmr (DMSO-$d_6$, 400 MHz): δ 13.82 (1H, s, C-8 OH), 10.50 (1H, br, C-2 OH), 7.73 (1H, t, J = 8.0 Hz, H-6), 7.65 (1H, d, J = 8.5 Hz, H-4), 7.49 (1H, d, J = 8.5 Hz, H-3), 7.07 (1H, dd, J = 0.8, 8.0 Hz, H-5), 6.80 (1H, dd, J = 0.8, 8.0 Hz, H-7), 3.84 (3H, s, $OCH_3$); $^{13}C$ nmr ($CDCl_3$-DMSO-$d_6$, 75 MHz): δ 180.56 (s, C-9), 170.16 (s, C-11), 160.76 (s, C-10a), 152.58 (s, C-4a), 152.25 (s, C-8), 148.00 (s, C-2), 135.83 (d, C-6), 125.94 (d, C-3), 119.04 (d, C-4), 117.21 (s, C-9a), 116.17 (s, C-1), 109.04 (d, C-5), 107.78 (s, C-8a), 106.83 (d, C-7), 51.85 (q, $OCH_3$); hrms m/z (relative intensity %) calc. for $C_{15}H_{10}O_6$ ($M^+$): 286.0477;

found: 286.0480 (32), 255 (20), 254 (100), 226 (12), 198 (3), 170 (3), 142 (5), 114 (3).

### 3.9 Acetylation of hydroxyvertixanthone

Acetic anhydride (2.5 mL) was added to a solution of hydroxyvertixanthone (16) (3.0 mg) in pyridine (0.5 mL). The solution was stirred at room temperature overnight. Toluene (5.0 mL) was added to the reaction mixture and the solvents were evaporated. Recrystallization from Skellysolve B gave acetylhydroxyvertixanthone (20) as colourless crystals (2.0 mg, 52% yield), mp 304-305°C; tlc: $R_f$ 0.40 (Skellysolve B-ethyl acetate 1:1); ir ($CHCl_3$, cast) $\upsilon_{max}$ $cm^{-1}$: 1771 (d, ester), 1734 (ester), 1658 (CO), 1622, 1214, 1195; $^1H$ nmr ($CDCl_3$, 300 MHz): $\delta$ 7.65 (1H, t, J = 8.5 Hz, H-6), 7.48 (2H, s, H-3, H-4), 7.35 (1H, dd, J =1.5, 8.5 Hz, H-5), 6.95 (1H, dd, J = 1.5, 8.5, Hz, H-7), 3.92 (3H, s, $OC\underline{H}_3$), 2.37 (3H, s, C-8 $OCOC\underline{H}_3$), 2.25 (3H, s, C-2 $OCOC\underline{H}_3$); $^{13}C$ nmr ($CDCl_3$, 75 MHz): $\delta$ 174.02 (s, C-9), 169.62 (s, C-8 $O\underline{C}OCH_3$), 168.77 (s, C-2 $O\underline{C}OCH_3$), 166.85 (s, C-11), 156.90 (s, C-10a), 152.75 (s, C-4a), 150.12 (s, C-8), 144.50 (s, C-2), 134.95 (d, C-6), 129.74 (d, C-3), 115.51 (s, C-9a), 125.75 (s, C-1), 113.50 (s, C-8a), 119.87 (d, C-5), 118.75 (d, C-4), 116.02 (d, C-7), 52.99 (q, $O\underline{C}H_3$), 21.16 (q, C-8 $OCO\underline{C}H_3$), 20.71 (q, C-2 $OCO\underline{C}H_3$); hrms m/z (relative intensity %) calc. for $C_{19}H_{14}O_8$ ($M^+$): 370.0688; found: 370.0699 (1), 328 (18), 286 (33), 255 (24), 254 (100), 226 (7), 198 (2), 170 (3), 142 (4), 114 (3).

### 3.10 Decarboxylation of hydroxyvertixanthone

Compound 20 (1.8 mg) was dissolved in chloroform, and 10% aqueous potassium carbonate (10 mL) was added to the solution. The mixture was stirred at

room temperature overnight. The reaction solution was neutralized with 1N HCl, then extracted with ethyl acetate. The extract was dried with anhydrous sodium sulfate and evaporated under reduced pressure. The residue was dissolved in quinoline (1.5 mL), heated to 240°C and refluxed for 25 minutes. The solution was cooled to room temperature and ethyl acetate (30.0 mL) was added. Quinoline was removed by extraction (three times) with water containing concentrated hydrochloric acid (pH = 2). The organic acid was extracted with aqueous 10% sodium hydroxide solution to give a yellow solution, and this was neutralized with concentrated hydrochloric acid, then extracted with ethyl acetate. The extract was dried with anhydrous sodium sulfate, and a yellow solid was obtained after evaporation of the solvents. Further purification was achieved by silica gel chromatography with 10% ethyl acetate in Skellysolve B. Crystallization from toluene gave yellow crystals of euxanthone (21) (1.0 mg, 90% yield), mp 236-238°C (236-238 °C, lit[43]); tlc: $R_f$ 0.61 (Skellysolve B-ethyl acetate 1:1); uv (95% EtOH, 0.8 mg/100 mL) $\lambda_{max}$ nm (log ε): 235 (4.03), 260 (4.12), 287 (3.43), 388 (3.41); ir (nujol) $\upsilon_{max}$ $cm^{-1}$: 3312 (br, OH), 1644 (CO, hydrogen-bonded), 1608, 1466, 1231; $^1H$ nmr ($CDCl_3$, 400 MHz): δ 12.61 (1H, s, C-1 O<u>H</u>), 8.82 (1H, br, C-7 O<u>H</u>), 7.62 (1H, d, J = 3.0 Hz, H-8), 7.59 (1H, t, J = 8.0 Hz, H-3), 7.42 (1H, d, J = 9.0 Hz, H-5), 7.34 (1H, dd, J = 3.0, 9.0, Hz, H-6), 6.93 (1H, dd, J = 0.8, 8.0 Hz, H-4), 6.79 (1H, dd, J = 0.8, 8.0 Hz, H-2); hrms m/z (relative intensity %) calc. for $C_{13}H_8O_4$) ($M^+$): 228.0422; found 228.0425 (100), 200 (9), 171 (3), 144 (4), 115 (6).

### 3.11 1,3,6,8-Tetrahydroxyanthraquinone (3)

An orange-red pigment was separated by repeated silica gel chromatography by elution with 20% ethyl acetate in methylene chloride. Crystallization from MeOH-

SKB gave crystalline 1,3,6,8-tetrahydroxyanthraquinone (3) (14.3 mg from C50 and 57.4 mg from C728), mp 290°C (decompose); tlc: $R_f$ 0.49 (Skellysolve B-ethyl acetate 3:4), $R_f$ 0.34 (methylene chloride-methanol 9.5:0.5); uv (95% EtOH, 1.0 mg/100 mL) $\lambda_{max}$ nm (log ε): 252 (4.13), 264 (4.16), 294 (4.32), 316 (3.97), 456 (3.18); ir ($CHCl_3$, cast) $\upsilon_{max}$ nm $cm^{-1}$: 3240 (br, OH), 1660 (CO) 1628 (CO, hydrogen-bonded), 1609, 1401, 1281, 1170, 760; $^1H$ nmr ($CDCl_3$-DMSO-$d_6$, 400 MHz): δ 12.24 (2H, s, 2xO<u>H</u>), 7.14 (2H, d, J = 2.0 Hz, H-4, H-5), 6.52 (2H, d, J = 2.0 Hz, H-2, H-7), 5.25 (2H, br, 2xO<u>H</u>); (acetone-$d_6$, 400 MHz) δ: 7.23 (2H, d, J = 2.0 Hz, H-4, H-5), 6.64 (2H, d, J = 2. 0 Hz, H-2, H-7); $^{13}C$ nmr (MeOH-$d_4$, 75 MHz): δ 188.50 (1C, s, C-9), 182.31 (1C, s, C-10), 165.43 (2C, s, C-3, C-6), 164.36 (2C, s, C-1, C-8), 134.78 (2C, s, C-4a, C-10a), 109.04 (2C, d, C-4, C-5), 108.44 (2C, s, C-8a, C-9a), 107.92 (2C, d, C-2, C-7); hrms m/z (relative intensity %) calc. for $C_{14}H_8O_6$ ($M^+$): 272.0320; found: 272.0321 (100), 244 (9), 216 (9), 188 (2), 160 (2), 122 (8), 91 (4), 77 (4), 63 (4).

### 3.12 Acetylation of 1,3,6,8-tetrahydroxyanthraquinone

Acetic anhydride (0.5 mL) was added into a stirred solution of 1,3,6,8-tetrahydroxyanthraquinone (3) (4.3 mg) in pyridine (1.0 mL). The reaction mixture was allowed to stir at room temperature for 12 hours. Toluene (5.0 mL) was added to the solution. Evaporation of the solvents gave a solid, which crystallized from Skellysolve B to give acetyl-1,3,6,8-tetrahydroxyanthraquinone (23) (3.0 mg, 50% yield), mp 191.5-193°C; ir ($CHCl_3$, cast) $\upsilon_{max}$ nm $cm^{-1}$: 1771, 1678, 1660, 1600, 1386, 1196, 1113; $^1H$ nmr ($CDCl_3$, 360 MHz): δ 7.96 (2H, d, J = 2.2 Hz, H-4, H-5), 7.27 (2H, d, J = 2.2 Hz, H-2, H-7), 2.43 (6H, s, 2xOCOC<u>H</u>$_3$), 2.35 (6H, s, 2xOCOC<u>H</u>$_3$); hrms m/z (relative intensity %) calc. for $C_{22}H_{16}O_{10}$ ($M^+$): 440.0743; found: 440.0729 (2), 398 (29), 356 (18), 314 (31), 272 (100), 243 (6).

### 3.13 1-Hydroxy-8-methoxyanthraquinone (24)

Chromatography over silica gel with 20% ethyl acetate in Skellysolve B or 2% methanol in methylene chloride gave an orange-yellow pigment. Crystallization from ethyl acetate-Skellysolve B gave 1-hydroxy-8-methoxyanthraquinone (**24**) (22.3 mg from C50 and 5.4 mg from C728), mp 182-183°C (185-186°C, lit[146]); tlc: $R_f$ 0.44 (Skellysolve B-ethyl acetate 3:2), $R_f$ 0.26 (Skellysolve B-benzene-methanol 1: 1: 0.2); uv (95% EtOH, 1.0 mg/ 100 mL) $\lambda_{max}$ nm (log $\varepsilon$): 253 (3.92), 277 (3.72), 412 (3.59); ir ($CHCl_3$, cast) $\upsilon_{max}$ nm $cm^{-1}$: 1668 (CO), 1628 (CO, hydrogen-bonded), 1580, 1480, 1450, 1440, 1350, 1280, 1240, 1070, 1020, 930, 840, 740; $^1H$ nmr ($CDCl_3$, 400 MHz): $\delta$ 12.96 (1H, s, OH), 7.96 (1H, dd, J = 1.3, 7.8 Hz, H-5), 7.77 (1H, dd, J = 1.3, 7.8 Hz, H-4), 7.74 (1H, t, J = 7.8 Hz, H-6), 7.60 (1H, t, J = 7.8 Hz, H-3), 7.35 (1H, dd, J = 1.3, 7.8 Hz, H-7), 7.29 (1H, dd, J = 1.3, 7.8 Hz, H-2), 4.04 (3H, s, $OC\underline{H}_3$); $^{13}C$ nmr ($CDCl_3$, 75 MHz): $\delta$ 188.86 (s, C-9), 182.69 (s, C-10), 162.55 (s, C-1), 160.97 (s, C-8), 135.87 (s, C-10a), 135.82 (d, C-3), 135.78 (d, C-6), 132.80 (s, C-4a), 124.73 (d, C-4), 120.93 (s, C-8a), 120.19 (d, C-5), 118.83 (d, C-2), 118.24 (d, C-7), 117.14 (s, C-9a), 56.69 (q, $O\underline{C}H_3$); hrms m/z (relative intensity %) calc. for $C_{15}H_{10}O_4$ ($M^+$): 254.0579; found: 254.0573 (100), 237 (16), 236 (59), 225 (22), 211 (4), 208 (89), 183 (5), 180 (26), 155 (23), 127 (22), 113 (10), 75 (21), 63 (25).

### 3.14 Methylation of 1-hydroxy-8-methoxyanthraquinone

1-Hydroxy-8-methoxyanthraquinone (**24**) (5.0 mg) was dissolved in acetone and saturated metholic potassium carbonate (1.0 mL) and methyl iodide (0.5 mL) were added. The solution changed colour from yellow to red when base was added. The mixture solution was refluxed for 12 hours, four further additions of $K_2CO_3$/MeOH

and $CH_3I$ were made at intervals until the solution did not change to red when base was added. Excess of $K_2CO_3$ was filtered and the solvents were evaporated under reduced pressure. The residue was dissolved in water and extracted with methylene chloride. The extract was concentrated to give a yellow solid which was crystallized from Skellysolve B containing a few drops of acetone. 1,8-Dimethoxyanthraquinone (**26**) was obtained as yellow needles (3.3 mg, 63% yield), mp 219-220°C (219°C, lit[147]); tlc: $R_f$ 0.22 (methylene chloride-methanol 9:1); ir ($CHCl_3$, cast) $\upsilon_{max}$ nm $cm^{-1}$: 1664 (d, CO), 1585, 1448, 1438, 1317, 1239, 977, 793; $^1H$ nmr ($CDCl_3$, 300 MHz): $\delta$ 7.68 (2H, dd, J = 1.5, 8.0 Hz, H-4, H-5), 7.57 (2H, t, J = 8.0 Hz, H-3, H-6), 7.24 (2H, dd, J = 1.5, 8.0 Hz, H-2, H-7), 3.94 (6H, s, 2xOC$\underline{H}_3$); $^{13}C$ nmr ($CDCl_3$, 75 MHz): $\delta$ 184.11 (1C, s, C-9), 182.93 (1C, s, C-10), 159.33 (2C, s, C-1, C-8), 134.85 (2C, s, C-4a, C-10a), 133.91 (2C, d, C-3, C-6), 124.14 (2C, s, C-8a, C-9a), 119.00 (2C, d, C-4, C-5), 118.14 (2C, d, C-2, C-7), 56.59 (2C, q, 2xO$\underline{C}H_3$); hrms m/z (relative intensity %) calc. for $C_{16}H_{12}O_4$ ($M^+$): 268.0736; found: 268.0737 (62), 253 (100), 236 (14), 225 (10), 208 (9), 180 (11), 152 (15), 139 (16), 76 (12).

### 3.15 5-Nonadecylresorcinol (28)

5-Nonadecylresorcinol (**28**) was isolated by chromatography over silica gel with 30% ethyl acetate in Skellysolve B. The white solids were precipitated from hexane (97.5 mg from C50 and 56.1 mg from C728), mp 90-91°C (96.5-97.5°C, lit[44]); tlc: $R_f$ 0.52 (Skellysolve B-ethyl acetate 3:2); uv (95% EtOH, 20 mg/100 mL) $\lambda_{max}$ nm (log $\varepsilon$): 275 (3.15), 28.1 (3.15); ir ($CHCl_3$, cast) $\upsilon_{max}$ $cm^{-1}$: 3320-3200 (br, OH), 1600, 1474, 1160, 826, 710; $^1H$ nmr ($CDCl_3$, 400 mHz): $\delta$ 6.22 (2H, d, J = 2.0 Hz, H-4, H-6), 6.15 (1H, t, J = 2.0 Hz, H-2), 4.73 (2H, s, 2xO$\underline{H}$), 2.46 (2H, t, J = 8.0 Hz, 2xH-1'), 1.50 (2H, m, 2xH-2'), 1.24 (32H, m, $(C\underline{H}_2)_{16}$), 0.86 (3H, t, J = 7.0 Hz, $CH_3$); $^{13}C$ nmr ($CDCl_3$, 75 MHz): $\delta$ 156.62 (2C, s, C-1, C-3), 146.22

(1C, s, C-5), 108.10 (2C, d, C-4, C-6), 100.12 (1C, d, C-2), 35.87 (1C, t, C-1'), 31.98 (1C, t, C-2'), 31.10 (1C, t, C-3'), 29.75 (10C, t, C-4'-C-13'), 29.74 (1C, t, C-14'), 29.65 (1C, t, C-15'), 29.41 (1C, t, C-16'), 29.34 (1C, t, C-17'), 22.73 (1C, t, C-18'), 14.16 (1C, q, C-19'); hrms m/z (relative intensity %) calc. for $C_{25}H_{44}O_2$ ($M^+$): 376.3343; found: 376.3339 (10), 348 (4), 166 (4), 137 (9), 124 (100), 123 (20); cims ($NH_3$) m/z (relative intensity %): 377 ($M^+ + 1$, 100).

### 3.16 2(3H)-Benzoxazolone (29)

The crude ether extract was extracted with 5% aqueous $Na_2CO_3$. The basic solution was neutralized and extracted with ether. The concentrated ether extract was separated by flash chromatography over silica gel. Elution with 30% ethyl acetate in Skellysolve B afforded 2(3H)-benzoxazolone (29) as colourless crystals (crystallization from Skellysolve B). It was also isolated from the ethyl acetate extract by chromatography as described above (24.0 mg from C50), mp 145.5-146°C (141-142°C, lit[148]); tlc: $R_f$ 0.46 (Skellysolve B-ethyl acetate 1:1); uv (95% EtOH, 1.0 mg/100 mL) $\lambda_{max}$ nm (log ε): 225 (3.95), 273 (3.70); ir (Nujol) $\upsilon_{max}$ $cm^{-1}$: 3220 (NH), 1770, 1732, 1620, 1478, 1250, 1140, 935, 738; $^1H$ nmr ($CDCl_3$, 400 MHz): δ 8.79 (1H, s, NH), 7.20 (1H, octet, J = 0.7, 1.7, 7.6 Hz, H-4), 7.15 (1H, sextet, J = 1.7, 7.6 Hz, H-6), 7.11 (1H, sextet, J = 1.7, 7.6 Hz, H-5), 7.06 (1H, octet, J = 0.7, 1.7, 7.6 Hz, H-7); $^{13}C$ nmr ($CDCl_3$, 75 MHz): δ 156.11 (s, C-2), 143.97 (s, C-1a), 129.47 (s, C-4a), 124.25 (d, C-5), 122.82 (d, C-6), 110.25 (d, C-4), 110.17 (d, C-7); hrms m/z (relative intensity %) calc. for $C_7H_5NO_2$ ($M^+$): 135.0357; found: 135.0306 (100), 106 (3), 91 (18), 79 (32), 64 (9), 52 (2); cims ($NH_3$) m/z (relative intensity %): 153 ($M^+ + 18$, 100).

### 3.17 Isoevernin aldehyde (30)

Compound **30**, isoevernin aldehyde, was isolated from the chromatographic fractions eluted with 15% ethyl acetate in Skellysolve B. Crystallization from ethyl acetate afforded compound **30** as pale brown needles (4.4 mg from C50), mp 196-197°C (196°C, lit[62]); tlc: $R_f$ 0.38 (Skellysolve B-ethyl acetate 1:1); uv (95% EtOH, 1.0 mg/100 mL) $\lambda_{max}$ nm (log ε): 280 (3.84), 313 (3.78); ir (KBr, pellet) $\upsilon_{max}$ $cm^{-1}$: 3140 (br), 2720, 1705, 1615, 1585, 1562, 1339; $^1H$ nmr ($CDCl_3$, 400 MHz) δ: 10.47 (1H, s, C<u>H</u>O), 6.34 (1H, d, J = 2.5 Hz, H-3), 6.24 (1H, d, J = 2.5 Hz, H-5), 4.27 (1H, br, O<u>H</u>), 3.87 (3H, s, OC<u>H</u>$_3$), 2.55 (3H, s, C<u>H</u>$_3$); $^{13}C$ nmr ($CDCl_3$, 75 MHz) δ: 191.77 (s, <u>C</u>HO), 167.57 (s, C-2), 165.68 (s, C-4), 145.94 (s, C-1), 117.10 (s, C-6), 112.32 (d, C-3), 97.58 (d, C-5), 56.27 (q, O<u>C</u>H$_3$), 22.27 (q, <u>C</u>H$_3$); hrms m/z (relative intensity %) calc. for $C_9H_{10}O_3$ ($M^+$): 166.0628; found: 166.0627 (100), 165 (84), 148 (12), 134 (11), 121 (10), 106 (18), 77 (11).

### 3.18 3,4-Dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone (31)

3,4-Dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone (**31**), isolated from the 30% ethyl acetate in Skellysolve B eluant, was crystallized from ethyl acetate to give colorless needles (15.8 mg from C50), mp 177-178°C; tlc: $R_f$ 0.12 (Skellysolve B-ethyl acetate 1:1); $[\alpha]_D$ -40° (c, 0.12, MeOH); uv (95% EtOH, 1.0mg / 100 mL) $\lambda_{max}$ nm (log ε): 259 (3.23); ir (KBr, pellet) $\upsilon_{max}$ $cm^{-1}$: 3600-2400, 1650, 1618, 1580, 1335, 790; $^1H$ nmr (MeOH-$d_4$, 360 MHz) δ: 7.55 (1H, t, J = 8.0 Hz, H-6), 7.14 (1H, dd, J = 2.0, 8.0 Hz, H-5), 6.86 (1H, dd, J= 2.0, 8.0 Hz, H-7), 4.62 (1H, d, J = 7.5 Hz, H-4), 4.06 (1H, m, H-3), 3.09 (1H, dd, J = 4.0, 17.5 Hz, H-2e), 2.69 (1H, dd, J = 7.5, 17.5 Hz, H-2a): $^{13}C$ nmr (MeOH-$d_4$, 75 MHz) δ: 204.36 (s, C-1), 163.23 (s, C-8), 145.83 (s, C-4a), 137.99 (d, C-6), 119.98 (d, C-5), 117.76 (d, C-

7), 116.75 (s, C-8a), 73.20 (d, C-4), 71.57 (d, C-3), 44.08 (t, C-2); hrms m/z (relative intensity %) calc. for $C_{10}H_{10}O_4$ ($M^+$): 194.0579; found: 194.0582 (80), 176 (15), 150 (70), 147 (20), 122 (63), 121 (100), 93 (17), 65 (26).

### 3.19 Vertipyronol (35)

Vertipyronol (35), a colorless oil, was separated by silica gel chromatography by elution with 95% ethyl acetate in Skellysolve B. The chromatographic fraction was purified by charcoal column (10-50% acetone in water) followed by silica gel flash chromatography (4% methanol in methylene chloride) (17.1 mg from C50), tlc: $R_f$ 0.29 (chloroform-methanol 1:9); $[\alpha]_D$ -1.58° (c, 0.19, $CHCl_3$); uv (95% EtOH, 2.0 mg/100 mL) $\lambda_{max}$ nm (log ε): 280 (3.74); ir ($CHCl_3$, cast) $\upsilon_{max}$ $cm^{-1}$: 3417 (br), 1699 (br), 1645, 1550, 1458, 1419, 1220, 1045, 817; $^1H$ nmr ($CDCl_3$, 360 MHz) δ: 7.24 (1H, s, H-6), 5.51 (1H, s, H-3), 3.90 (3H, s, $OC\underline{H}_3$), 3.75 (1H, dd, J = 6.0, 10.5 Hz, H-8), 3.65 (1H, dd, J = 6.0, 10.5 Hz, H-8), 2.95 (1H, tq, J = 6.0, 7.5 Hz, H-7), 2.15 (1H, br, O<u>H</u>), 1.26 (3H, d, J = 7.5 Hz, $C\underline{H}_3$); $^{13}C$ nmr ($CDCl_3$, 75 MHz) δ: 169.97 (s, C-4), 164.42 (s, C-2), 148.65 (d, C-6), 117.08 (s, C-5), 90.00 (d, C-3), 65.75 (t, C-8), 56.08 (q, $O\underline{C}H_3$), 32.48 (d, C-7), 15.85 (q, $\underline{C}H_3$); hrms m/z (relative intensity %) calc. for $C_9H_{12}O_4$ ($M^+$): 184.0736; found: 184.0737 (18), 154 (11), 153 (100), 125 (6), 97 (6), 93 (5).

### 3.20 Acetylation of vertipyronol

Vertipyronol (2.0 mg) was treated with acetic anhydride in pyridine at room temperature for 12 hours. Work up in the usual way gave acetylvertipyronol, compound 37, as a yellowish oil (1.9 mg, 77% yield), $[\alpha]_D$ -2.63° (c, 0.19, $CHCl_3$); ir ($CHCl_3$, cast) $\upsilon_{max}$ $cm^{-1}$: 1745-1734, 1648, 1555, 1458, 1421, 1210, 1043, 817;

$^1$H nmr ($CDCl_3$, 400 MHz) δ: 7.22 (1H, s, H-6), 5.56 (1H, s, H-3), 4.15 (1H, dd, J = 6.0, 10.5 Hz, H-8), 4.08 (1H, dd, J = 6.0, 10.5 Hz, H-8), 3.85 (3H, s, $OC\underline{H}_3$), 3.05 (3H, tq, J = 6.0, 7.5, Hz, H-7), 2.04 (3H, s, $OCOC\underline{H}_3$), 1.22 (3H, d, J = 7.5, Hz, $C\underline{H}_3$); hrms m/z (relative intensity %) calc. for $C_{11}H_{14}O_5$ ($M^+$): 226.0841; found: 226.0842 (10), 166 (69), 154 (16), 153 (100), 151 (13), 125 (9), 93 (8).

3.21 Diels-Alder reaction of vertipyronol

N-Phenylmaleimide (14.4 mg, about 3 eq. excess) was added to a stirred solution of vertipyronol (**35**) (5.0 mg) in xylene. The mixture was refluxed at 140°C for 12 hours. The solvents were evaporated and the residue was separated by repeated silica gel chromatography (eluant: 5% methanol in methylene chloride). Evaporation of the solvent gave a yellowish solid, which was further purified by precipitation from ethyl acetate and hexane. The diadduct, compound **38**, was obtained as a yellowish solid (5.6 mg, 42% yield), $[\alpha]_D$ -3.06° (c, 0.36, $CHCl_3$); ir ($CHCl_3$, cast) $\upsilon_{max}$ $cm^{-1}$: 3560-3490, 1715, 1499, 1380, 1219, 1193, 746, 690; $^1$H nmr ($CDCl_3$, 360 MHz) δ: 7.48 (8H, m, aromatic hydrogens), 7.42 (2H, m, aromatic hydrogens), 4.25 (1H, t, J = 3.0 Hz, H-1), 3.91 (1H, t, J = 3.0 Hz, H-4), 3.67 (3H, s, $OC\underline{H}_3$), 3.40 (2H, t, J = 6.5 Hz, 2xH-1'), 3.23 (2H, dd, J = 3.0, 8.0 Hz, H-6, H-7), 3.14 (2H, dd, J = 3.0, 8.0 Hz, H-5, H-8), 2.73 (1H, tq, J = 6.5, 7.0 Hz, H-2'), 0.86 (3H, d, J = 7.0 Hz, $C\underline{H}_3$); hrms m/z (relative intensity %) calc. for $C_{28}H_{26}N_2O_6$ ($M^+$): 486.1792; found: 486.1775 (1), 456 (100), 455 (90), 442 (19), 441 (21), 174 (30), 136 (10), 135 (18), 119 (10), 105 (9), 91 (13), 77 (18).

### 3.22 Vertipyronediol (39)

Vertipyronediol was isolated from the crude ethyl acetate extract by charcoal chromatography (10-50% acetone in water) followed by silica gel flash chromatography (5% methanol in methylene chloride). Crystallization from acetone-Skellysolve B gave compound **39** as colorless crystals (6.5 mg from C50), mp 128-129°C; $[\alpha]_D$ -5.0° (c, 0.20, $CHCl_3$); tlc: $R_f$ 0.50 (chloroform-methanol 2:8); uv (95% EtOH, 1.0 mg/100 mL) $\lambda_{max}$ nm (log ε): 279 (3.51); ir ($CHCl_3$, cast) $\upsilon_{max}$ $cm^{-1}$: 3390 (br), 3291, 1711 (br), 1638, 1546, 1347, 1157, 1064, 828; $^1H$ nmr ($CDCl_3$, 360 MHz) δ: 7.65 (1H, s, H-6), 5.56 (1H, s, H-3), 3.98 (1H, d, J= 11.0 Hz, H-8), 3.90 (3H, s, $OC\underline{H}_3$), 3.64 (1H, d J = 11.0 Hz, H-8), 3.18 (1H, br, O<u>H</u>), 1.75 (1H, br, O<u>H</u>), 1.52 (3H, s, $C\underline{H}_3$); $^{13}C$ nmr ($CD_3OD$, 75 MHz) δ: 171.64 (s, C-4), 167.41 (s, C-2), 151.86 (d, C-6), 120.89 (s, C-5), 90.55 (d, C-3), 73.67 (s, C-7), 69.16 (t, C-8), 56.65 (q, $O\underline{C}H_3$), 24.50 (q, $\underline{C}H_3$); hrms m/z (relative intensity %) calc. for $C_9H_{12}O_5$ ($M^+$): 200.0685; found: 200.0684 (4), 170 (8), 169 (100), 151 (13), 127 (99), 99 (10); cims ($NH_3$) m/z (relative intensity %): 218 ($M^+$ + 18, 96), 201 ($M^+$ + 1, 100).

### 3.23 Acetylation of vertipyronediol

Vertipyronediol (**39**) (1.0 mg) was treated with acetic anhydride in pyridine at room temperature for 12 hours. Work up in the usual manner gave monoacetyl vertipyronediol, compound **40**, as colorless crystals (1.0 mg, 83% yield). $[\alpha]_D$ -10.0° (c, 0.08, $CHCl_3$); ir ($CHCl_3$, cast) $\upsilon_{max}$ $cm^{-1}$: 3420 (br), 1739, 1716, 1642, 1545. 1225, 1043, 1001; $^1H$ nmr ($CDCl_3$, 400 MHz) δ: 7.61 (1H, s, H-6), 5.58 (1H, s, H-3), 4.39 (1H, d, J = 11.5 Hz, H-8), 4.27 (1H, d, J = 11.5 Hz, H-8), 3.88 (3H, s, $OC\underline{H}_3$), 3.01 (1H, br, O<u>H</u>), 2.06 (3H, s, $OCOC\underline{H}_3$), 1.50 (3H, s, $C\underline{H}_3$); hrms m/z

(relative intensity %) calc. for $C_{11}H_{14}O_6$ ($M^+$): 242.0790; found: 242.0792 (7), 182 (3), 170 (9), 169 (100), 153 (6), 151 (7), 127 (52).

### 3.24 1,8-Dihydroxyanthraquinone (41)

The crude ether and ethyl acetate extracts were separated by silica gel flash chromatography (eluant: 5% ethyl acetate in Skellysolve B). The chromatographic fractions were further purified by extraction with 1% aqueous NaOH solution, neutralized with 1N HCl, and then extracted with methylene chloride. Evaporation of the solvents and crystallization from ethyl acetate-Skellysolve B gave yellow needles of 1,8-dihydroxyanthraquinone (**42**) (8.1 mg from C728), mp 193-194°C (193°C, lit[147]); tlc: $R_f$ 0.45 (Skellysolve B-ethyl acetate 3:1); uv (95% EtOH, 1.0 mg/100 mL) $\lambda_{max}$ nm (log $\epsilon$): 252 (4.12), 283 (3.85), 430 (3.76); ir ($CHCl_3$, cast) $\upsilon_{max}$ $cm^{-1}$: 3100 (br), 1664, 1628, 1445, 1281, 1190, 743; $^1H$ nmr ($CDCl_3$, 400 MHz) $\delta$: 12.08 (2H, s, 2xOH), 7.84 (2H, dd, J = 1.8, 7.5 Hz, H-4, H-5), 7.69 (2H, t, J = 7.5 Hz, H-3, H-6), 7.30 (2H, dd, J = 1.8, 7.5 Hz, H-2, H-7); $^{13}C$ nmr ($CDCl_3$, 75 MHz) $\delta$ : 193.16 (1C, s, C-9), 181.77 (1C, s, C-10), 162.62 (2C, s, C-1, C-8), 137.31 (2C, d, C-3, C-6), 133.69 (2C, s, C-4a, C-10a), 124.68 (2C, d, C-4, C-5), 120.09 (2C, d, C-2, C-7), 115.93 (2C, s, C-8a, C-9a); hrms m/z (relative intensity %) calc. for $C_{14}H_8O_4$ ($M^+$): 240.0422; found: 240.0422 (100), 212 (14), 184 (11), 156 (2), 128 (5).

### 3.25 Mycoxanthone (42)

Mycoxanthone (**42**) was isolated as yellow crystals from ethyl acetate-Skellysolve B by silica gel flash chromatography (eluant: 10% ethyl acetate in Skellysolve B) (4.4 mg from C728), mp 221-222°C (222-223, lit[94]); tlc: $R_f$ 0.23

(Skellysolve B-ethyl acetate 3:1); uv (95% EtOH, 1.5 mg/100 mL) $\lambda_{max}$ nm (log ε): 235 (5.13), 261 (5.16), 290 (4.60), 385 (4.52); ir (nujol) $\upsilon_{max}$ $cm^{-1}$: 3300 (br), 1750, 1651, 1604, 1591, 1490, 1464, 1283, 1251, 1184, 1087; $^1H$ nmr ($CDCl_3$, 360 MHz) δ: 12.25 (1H, s, OH), 7.58 (1H, t, J = 8.0 Hz, H-6), 7.54 (1H, d, J = 9.2 Hz, H-4), 7.42 (1H, d, J = 9.2 Hz, H-3), 6.90 (1H, dd, J = 1.2, 8.0 Hz, H-5), 6.78 (1H, dd, J = 1.2, 8.0 Hz, H-7), 4.05 (3H, s, $OCOCH_3$), 3.92 (3H, s, $OCH_3$); $^{13}C$ nmr ($CDCl_3$, 75 MHz) δ: 181.24 (s, C-9), 167.60 (s, C-11), 161.86 (s, C-10a), 156.09 (s, C-4a), 152.51 (s, C-8), 137.12 (d, C-6), 120.50 (s, C-1), 120.07 (d, C-3), 120.00 (d, C-4), 118.26 (s, C-9a), 110.56 (d, C-5), 108.66 (s, C-8a), 106.84 (d, C-7), 57.05 (q, $OCOCH_3$), 53.12 (q, $OCH_3$); hrms m/z (relative intensity %) calc. for $C_{16}H_{12}O_6$ ($M^+$): 300.0634; found: 300.0633 (45), 269 (19), 268 (100), 254 (4), 240 (10), 225 (5).

4. Biological Studies of Crude Extracts and Metabolites of *Verticicladiella* sp. C50 and C728

4.1 Inhibition of water conduction bioassay

The mixtures of four crude extracts of *Verticicladiella* sp. C50 or C728 and nine pure metabolites: 1,8-dihydroxyanthraquinone (**3**), 1,8-dimethoxynaphthalene (**6**), vertixanthone (**11**), hydroxyvertixanthone (**16**), 1-hydroxy-8-methoxy-anthraquinone (**24**), 5-*n*-nonadecylresorinol (**28**), 2(3H)-benzoxazolone (**29**), 1,8-dimethoxyanthraquinone (**41**), and mycoxanthone (**42**) were subjected to the inhibition of water conduction bioassay. One month old pine seedlings were cut with a sharp razor-blade under water. Three seedlings were placed in one 1 dram vial which containing 1 mL of 0.1% aqueous test solution, and the vials were kept at room temperature for 24 hours (approximately 12 hours light). The seedlings were

transfered from the test solution to the 0.1% acid Fuchsin dye solution and after 24 hours the uptake of dye was measured by observing the movement of the dye up through the seedlings.

4.2 Antibiotic screening: Agar diffusion using aqueous test solutions

The susceptibility of four micro-organisms (Table 28) to antibiotic substances present in *Verticicladiella* extracts was determined by agar diffusion using an aqueous test solution. Since the pure metabolites do not dissolve well in aqueous solution, the sodium salts of the anthraquinone and xanthone compounds were used for the test. Presterilized vessels (5/16" OD x 5/16" long cylinder cut from 304 stainless steel tubes) were placed onto the Mueller Hinton agar plates which had been swabbed with the test organism. 5% Aqueous test solution (100 µL) was placed into each vessel. The plates were incubated at 37°C . Inhibition zone diameters (mm) were recorded after 18 hours.

5. Biosynthetic Studies of Metabolites Produced by *Verticicladiella* sp. C50 and C728

5.1 Incorporation of [1-$^{13}$C]-labelled acetate.

*Verticicladiella* sp. C50 was grown in liquid still culture (20 mL) on a medium of 10% V-8 juice and 1% glucose in five 2 L Fernbach flasks (1 liter of medium / flask). After 12 days of growth, a sterile solution of sodium [1-$^{13}$C] acetate (0.082 g, 1 mmol) in distilled water (5 mL), was injected into each flask. After a further 7 and then 14 days, an additional 1.0 mmol of labelled acetate was injected into each flask. After a total of six weeks of growth the mycelium was removed by filtration (through

cheesecloth), washed with cold methanol, and air dried. The dried mycelium was extracted successively in a Soxhlet extractor with Skellysolve B (24 h), methylene chloride (24 h), and ethyl acetate (24 h). The crude extracts were evaporated to dryness *in vacuo* at room temperature. Each crude extract was separated by silica gel flash chromatography. 1,8-Dimethoxynaphthalene (**6**) was isolated from the Skellysolve B extract. Vertixanthone (**11**), hydroxyvertixanthone (**16**) and compound **6** were isolated from the methylene chloride and ethyl acetate extracts.

The culture broth decanted from the mycelium was concentrated *in vacuo* to ca. 500 mL and continuously extracted with Skellysolve B (twice, 24 h each) and ethyl acetate (twice, 24 h each). The crude extracts were dried and separated by flash chromatography over silica gel. Compound **6** was isolated from both Skellysolve B and ethyl acetate extracts, while 3,4-dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone (**31**) was isolated from the ethyl acetate extract.

5.2 Incorporation of [1,2-$^{13}$C]-labelled acetate

*Verticicladiella* sp. C50 was grown on solid medium of rye in two autoclavable plastic bags as described in Section 1. After 7 days of growth, a sterile solution of sodium [1,2-$^{13}$C] acetate (0.246 g, 3 mmol) in distilled water (15 mL) was injected into each bag. Just before and after injection, the bag was shaken several times. After six weeks of growth, the culture was extracted successively in a Soxhlet extractor with Skellysolve B (24 h), methylene chloride (24 h), and ethyl acetate (24 h). Each crude extract was separated by flash chromatography. Compound **6** was isolated from the Skellysolve B extract, compound **31**, 1-hydroxy-8-methoxy-anthraquinone (**24**) and hydroxyvertixanthone (**16**) were isolated from the methylene chloride extract, while 1,3,6,8-tetrahydroxyanthraquinone (**3**) and compound **31** were isolated from the ethyl acetate extract.

## 6. [1-$^{13}$C]-Acetate Labelled Metabolites

### 6.1 1,8-Dimethoxynaphthalene (6)

1,8-Dimethoxynaphthalene (**6**) (44.3 mg from mycelium extract and 30.0 mg from broth extract, about 6% incorporation), $^{13}$C nmr ($CDCl_3$, 75 MHz, BB) enriched signals δ: 157.08 (C-1 and C-8), 137.37 (C-4a), 126.34 (C-3 and C-6); natural abundance signals δ: 120.83 (C-4 and C-5), 117.57 (C-8a), 106.16 (C-2 and C-7), 56.43 (2x$\underline{C}H_3$).

### 6.2 3,4-Dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone (31)

3,4-Dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone (**31**) (22.6 mg from broth extract, about 6% incorporation), $^{13}$C nmr ($CD_3OD$, 75 MHz, BB) enriched signals δ: 204. 11 (C-1), 163.24 (C-8), 145.82 (C-4a), 137.99 (C-6), 71.68 (C-3); natural abundance signals δ: 120.01 (C-5), 117.77 (C-7), 116.74 (C-8a), 73.24 (C-4), 44.31 (C-2).

### 6.3 Vertixanthone (11)

Vertixanthone (**11**) (3.1 mg from mycelium extract, about 7% incorporation), $^{13}$C nmr ($CDCl_3$, 75 MHz, BB) enriched signals δ: 181.06 (C-9), 161.86 (C-10a), 156.11 (C-4a), 155.85 (C-8), 137.19 (C-6), 135.08 (C-3), 133.73 (C-1); natural abundance signals δ: 169.59 (C-11), 122.72 (C-2), 119.52 (C-4), 117.56 (C-9a), 110.99 (C-5), 109.04 (C-8a), 106.93 (C-7), 53.17 (O$\underline{C}H_3$).

6.4 Hydroxyvertixanthone (16)

Hydroxyvertixanthone (16) (2.0 mg from mycelium extract, about 9% incorporation), $^{13}C$ nmr ($CD_3OD$, 75 MHz, BB) enriched signals δ: 182.53 (C-9), 162.77 (C-10a), 157.52 (C-4a), 150.99 (C-8), 138.24 (C-6), 126.17 (C-3), 118.77 (C-1); natural abundance signals δ: 170.01 (C-11), 152.32 (C-2), 121.29 (C-4), 119.05 (C-9a), 111.07 (C-5), 108.04 (C-8a), 108.03 (C-7), 53.29 ($O\underline{C}H_3$).

7. [1,2-$^{13}C$]-Acetate Labelled Metabolites

7.1 1,3,6,8-Tetrahydroxyanthraquinone (3)

1,3,6,8-Tetrahydroxyanthraquinone (3) (98.8 mg from ethyl acetate extract, about 0.1% incorporation), $^{13}C$ nmr (DMSO-$d_6$, 75 MHz, BB) $^1J_{cc}$ (δ): C-1 (164.21), C-2 (108.07), 61.9 Hz; C-3 (165.03), C-4 (108.69), 62.5 Hz; C-4a (134.90), C-10 (181.07), 60.5 Hz; C-5 (108.69), C-10a (134.90), 61.8 Hz; C-6 (165.03), C-7 (108.07), 62.5 Hz; C-8 (164. 21), C-8a (108.07), 61.5 Hz; C-9 (188.55), C-9a (108.07), 61.5 Hz.

7.2 1,8-Dimethoxynaphthalene (6)

1,8-Dimethoxynaphthalene (6) (582.4 mg from Skellysolve B extract, about 0.1% incorporation), $^{13}C$ nmr ($CDCl_3$, 75 MHz, BB) $^1J_{cc}$ (δ): C-1 (157.10), C-2 (106.19), 70.1 Hz; C-3 (126.53), C-4 (120.83), 59.7 Hz; C-4a (137.39), C-5 (120.83), 55.7 Hz; C-6 (126.35), C-7 (106.19), 55.7 Hz; C-8 (157.10), C-8a (117.60), 67.7 Hz.

## 7.3 3,4-Dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone (31)

3,4-Dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone (31) (15.8 mg from methylene chloride extract and 21.0 mg from ethyl acetate extract, about 0.2% incorporation), $^{13}C$ nmr ($CD_3OD$, 75 MHz, BB) $^1J_{cc}$ ($\delta$): C-1 (204.33), C-8a (116.74), 57.4 Hz; C-2 (44.32), C-3 (71.67), 39.7 Hz; C-4 (73.25), C-4a (145.83), 40.8 Hz; C-5 (120.07), C-6 (137.99), 59.8 Hz; C-7 (117.76), C-8 (163.28), 58.2 Hz.

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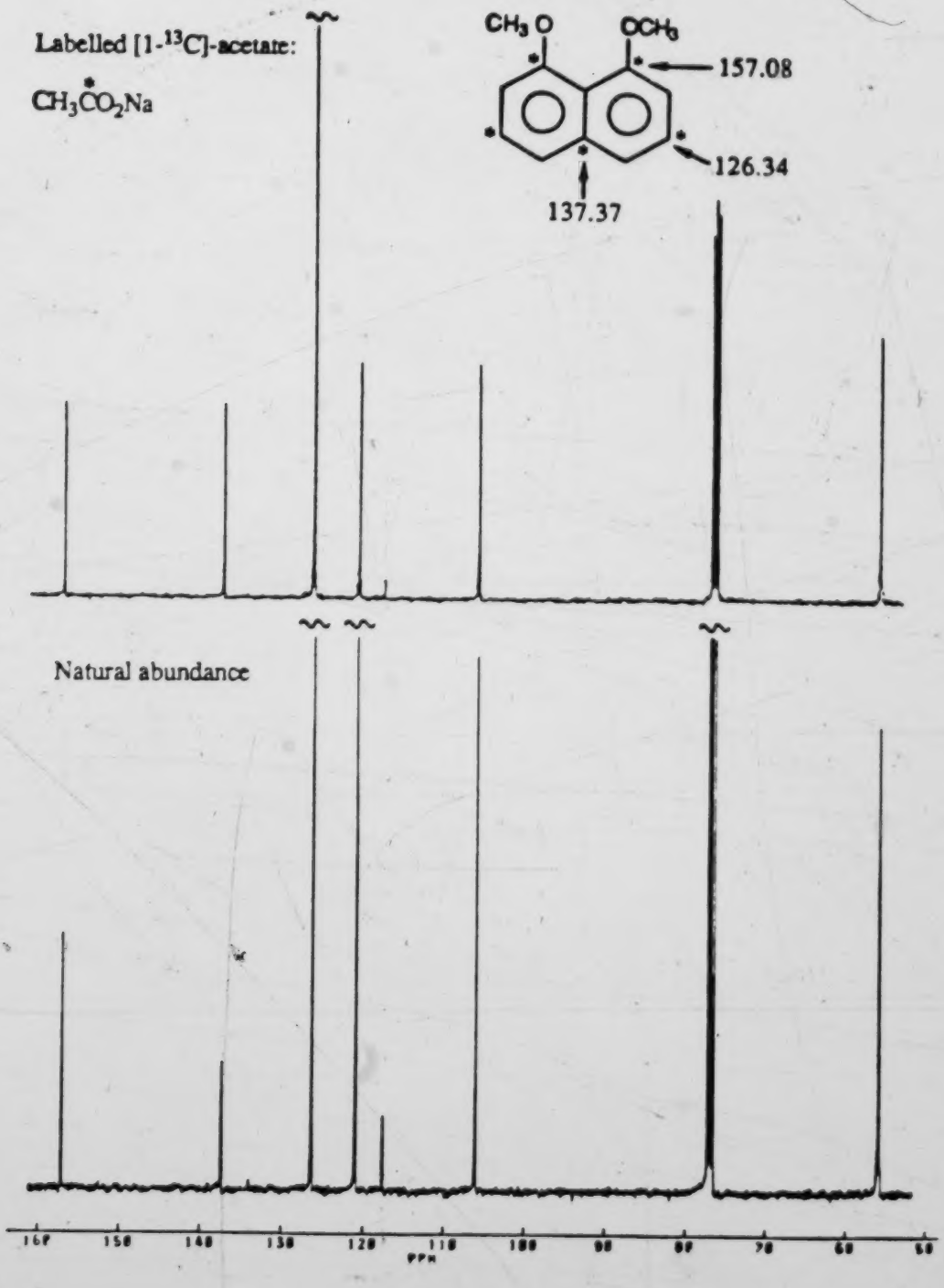
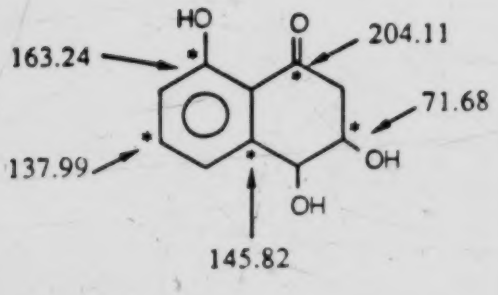
## V. APPENDICES

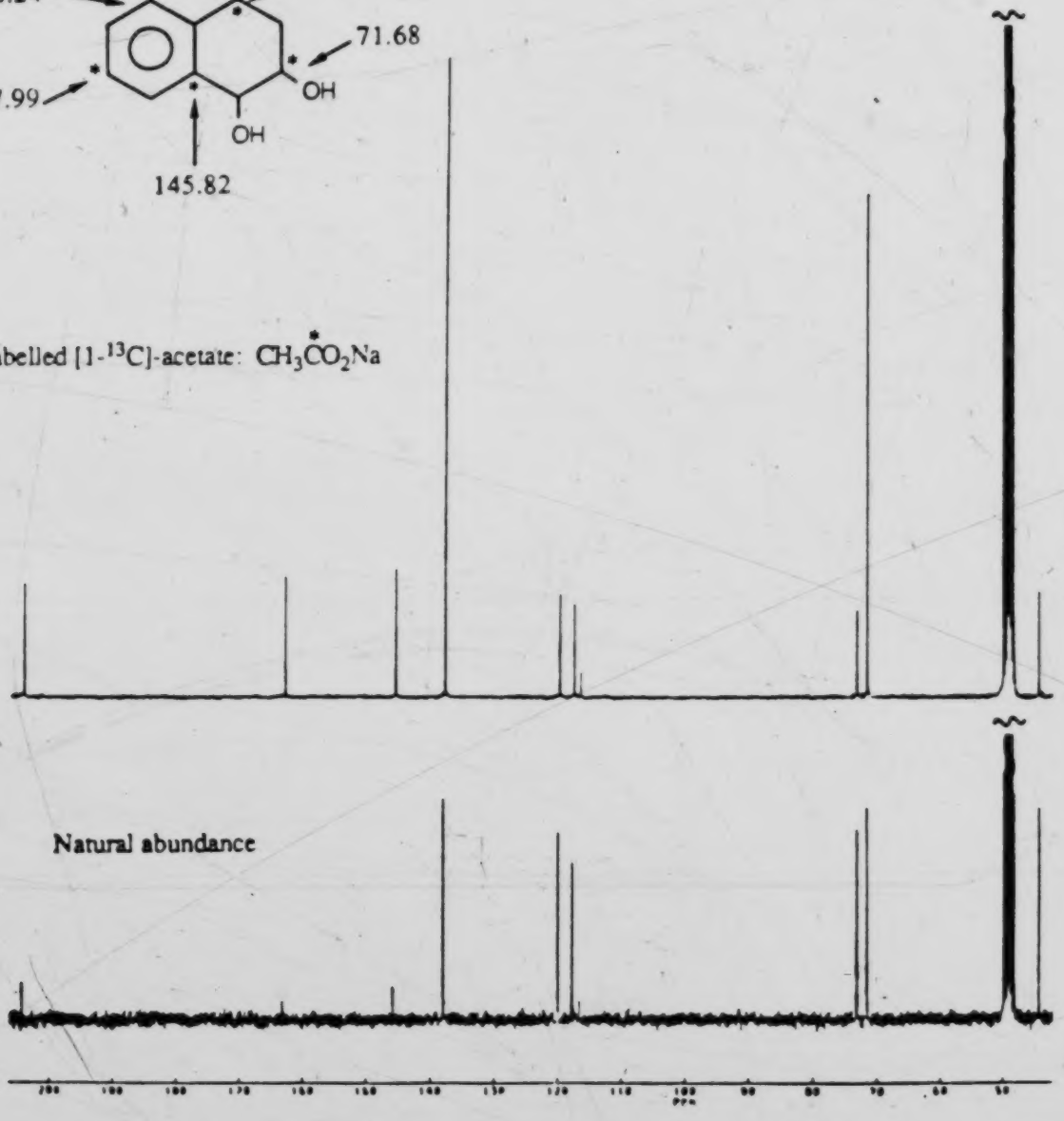
$^{13}C$ nmr spectra of 1,8-dimethoxynaphthalene ($CDCl_3$, 75 MHz, BB)



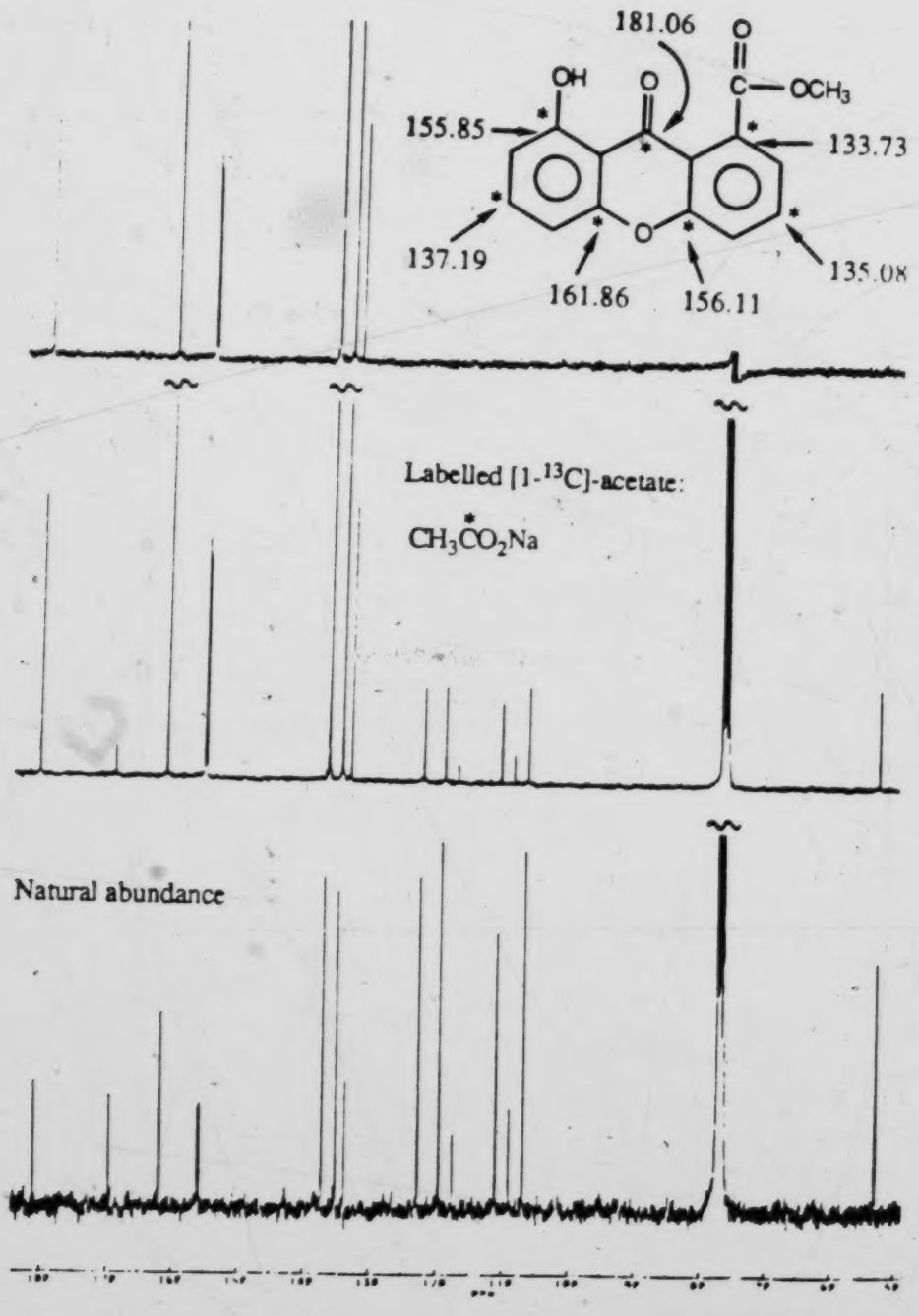
$^{13}C$ nmr spectra of 3,4-dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone ($CD_3OD$, 75 MHz, BB)



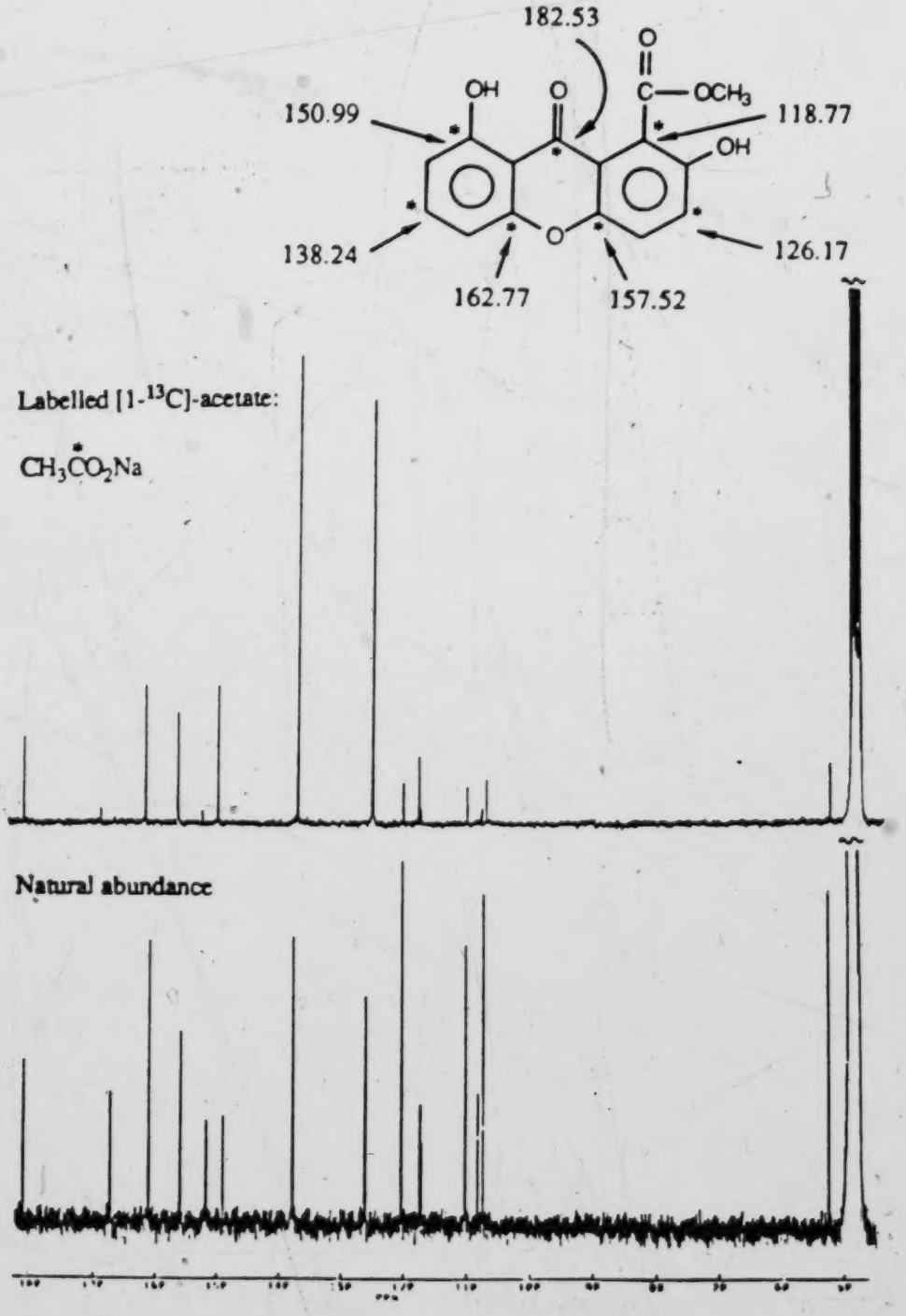

Labelled [1-$^{13}C$]-acetate: $CH_3\overset{*}{C}O_2Na$



$^{13}C$ nmr spectra of vertixanthone ($CDCl_3$, 75 MHz, BB)



$^{13}C$ nmr spectra of hydroxyvertixanthone ($CD_3OD$, 75 MHz, BB)



$^1H$ nmr spectrum of 2(3H)-benzoxazolone ($CDCl_3$, 400 MHz)

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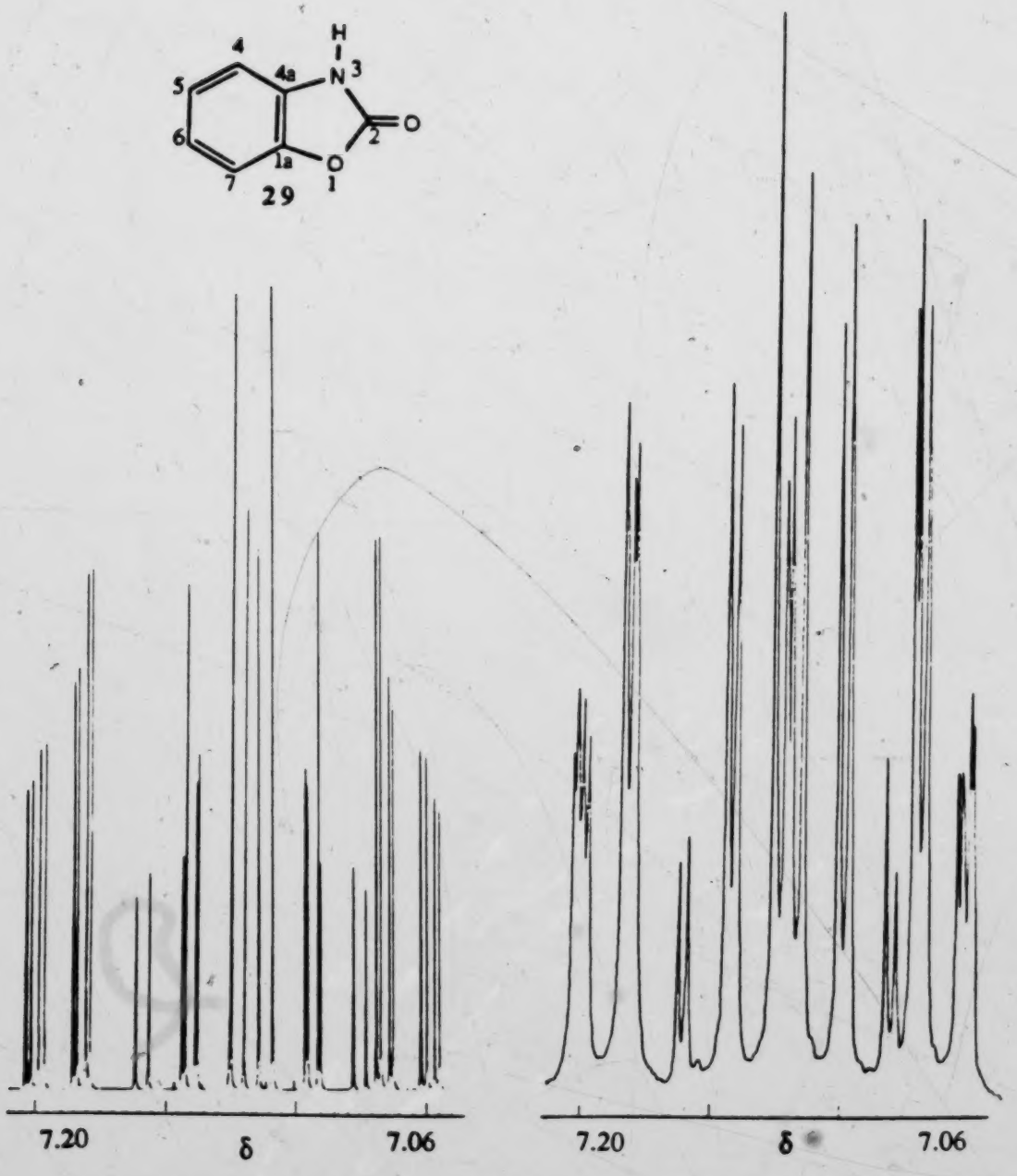


Calculated spectrum (PANIC)

Observed spectrum ($^1H$ nmr , $CDCl_3$, 400 MHz)